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Docket No.: 229752001500
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Patent Application of:
Charles A. COLLYER et al.

Application No.: 09/980,370

Confirmation No.: 1330

International

Art Unit: 1653

Filing Date: May 26, 2000

For: METHOD OF PROPHYLAXIS AND
TREATMENT AND AGENTS USEFUL FOR
SAME

Examiner: R. Mondesi

SUPPLEMENTAL AMENDMENT

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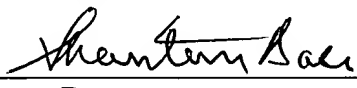
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MORRISON & FOERSTER LLP

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(650) 813-5995



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Short communication

Isolation and characterization of a hemin-binding cell envelope protein from *Porphyromonas gingivalis*

Sung-Jo Kim, L. Chu, and S. C. Holt*

Department of Microbiology, University of Texas Health Center at San Antonio,
San Antonio, TX 78284, U.S.A.

(Received November 30, 1995; revised manuscript accepted February 24, 1996)

Kim, S.-J. (Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78284, U.S.A.), L. Chu and S. C. Holt. Isolation and characterization of a hemin-binding cell envelope protein from *Porphyromonas gingivalis*. *Microbial Pathogenesis* 1996; 21, 65-70.

A 30 kDa (heated 24 kDa) hemin-binding protein whose expression is both hemin and iron regulated was identified and purified in *Porphyromonas gingivalis* 381. A strong hemin-binding function was found by LDS-PAGE and TMBZ staining when cells were grown under hemin (iron)-limited conditions. N-terminal amino acid sequence analysis of CNBr-digested 24 kDa hemin binding protein revealed that this protein belongs to a new, so far undescribed hemin-binding class of proteins.

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Key words: Hemin; *Porphyromonas gingivalis*; TMBZ; outer membranes.

Introduction

While there are several studies which have dealt with the expression of hemin-regulated proteins in the oral pathogen, *Porphyromonas gingivalis*,¹⁻⁵ there are few studies which have demonstrated actual hemin-binding. Smalley *et al.*⁵ identified a major tetramethylbenzidine (TMBZ) staining 32 kDa (unheated) protein in hemin-limited *P. gingivalis* W50, while Grenier⁶ demonstrated that the hemin-binding property of *P. gingivalis* ATCC 33277 is mediated by the lipopolysaccharides, particularly the Lipid A region. In the study presented here, we have identified, purified, and characterized a putative hemin binding protein in *P. gingivalis* 381.

Results

Identification of hemin-binding protein

In Fig. 1(a), the upregulation of the 56 and 30 kDa proteins (unheated) is very clear. TMBZ staining [Fig. 1(b)] revealed the only protein to bind the stain was at 30 kDa when cells were grown under hemin(iron) restriction. Note in Fig. 1(b) that

* To whom correspondence and reprint requests should be addressed.

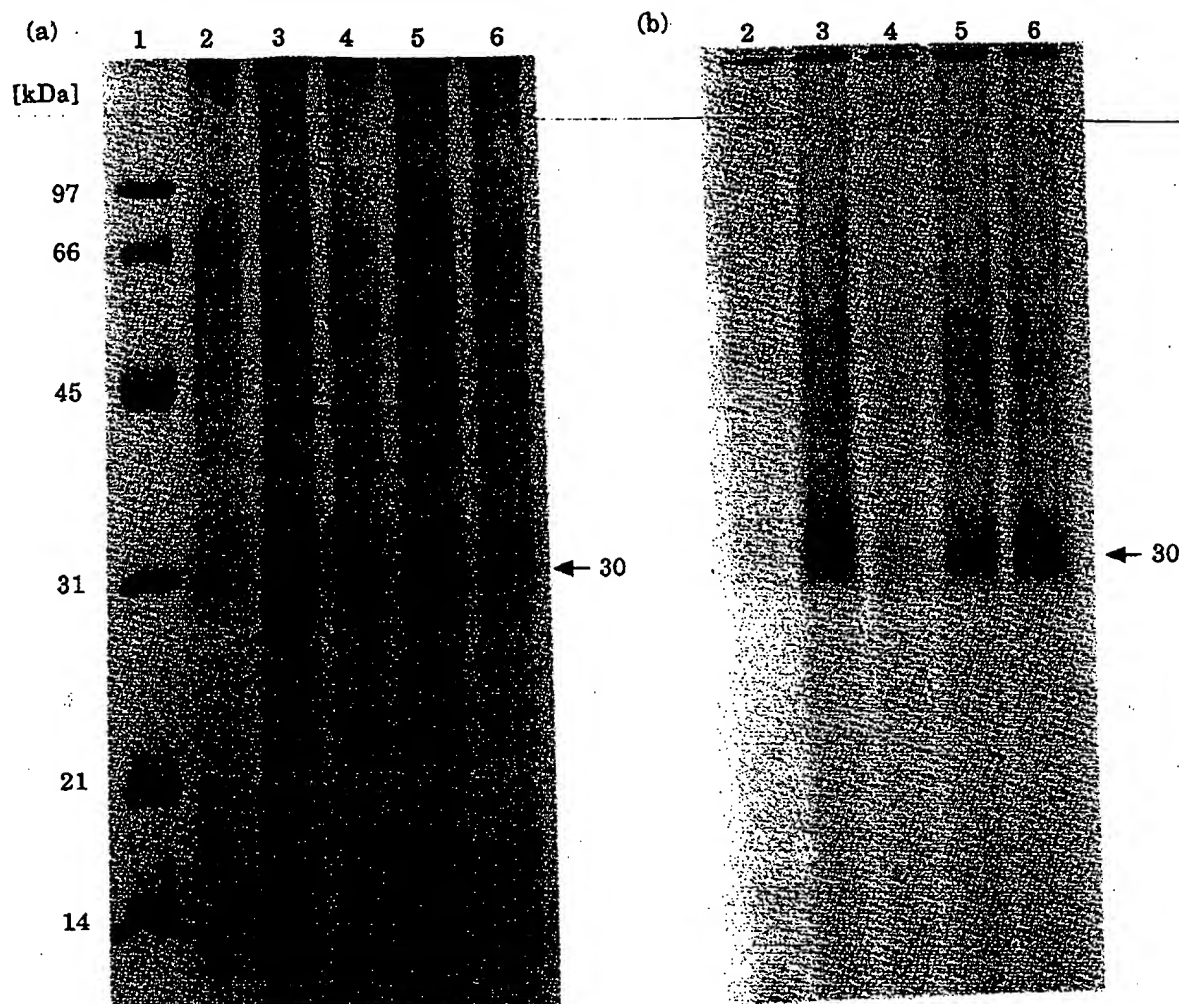


Fig. 1. Coomassie Brilliant blue stained (a) and tetramethylbenzidine (TMBZ) stained (b) LDS-PAGE of cell envelopes from *P. gingivalis* 381. Protein (70 μ g) was applied to each lane following incubation with hemin. Lane 1, low mol.wt. standards; lane 2, cells grown + 7.7 μ M hemin; lane 3 passage 5; lane 4, cells grown + 200 μ M BPD; lane 5, cells grown + 300 μ M BPD; lane 6, cells grown + 400 μ M BPD.

Fig. 2. SDS protein from 3, 1% CHAPS protein; lane

with increasing iron restriction (i.e. 300, 400 μ M BPD), there was increased TMBZ binding. A series of very lightly staining TMBZ bands might correspond to LPS.

Purification of hemin-binding protein

The 30 kDa *P. gingivalis* 381 hemin-binding protein was sequentially purified from the unheated cell envelopes (Fig. 2). In Fig. 2, lane 2, the cell envelope fraction contained numerous cell envelope proteins, including LPS-associated proteins. 1% CHAPS solubilization resulted in the removal of a large number of membrane proteins and associated LPS. The 30 kDa protein in Fig. 2, lane 3 was the major protein. The 30 kDa protein was isolated from the SDS-PAGE gels of the 1% CHAPS-soluble fraction (Fig. 2, lane 4). This protein was aggregated with proteins at 24 and 56 kDa. Heating of the isolated 30 kDa protein resulted in the appearance of the 24 kDa protein and several other minor and weakly staining proteins (Fig. 2, lane 5). The 24 kDa protein was isolated from the SDS-PAGE of this heated 30 kDa protein (Fig 2, lane 6). The resulting 24 kDa protein was isolated from the gel as a single protein band, with no contaminating proteins even when the gels were overloaded with large amounts of purified protein (data not shown).

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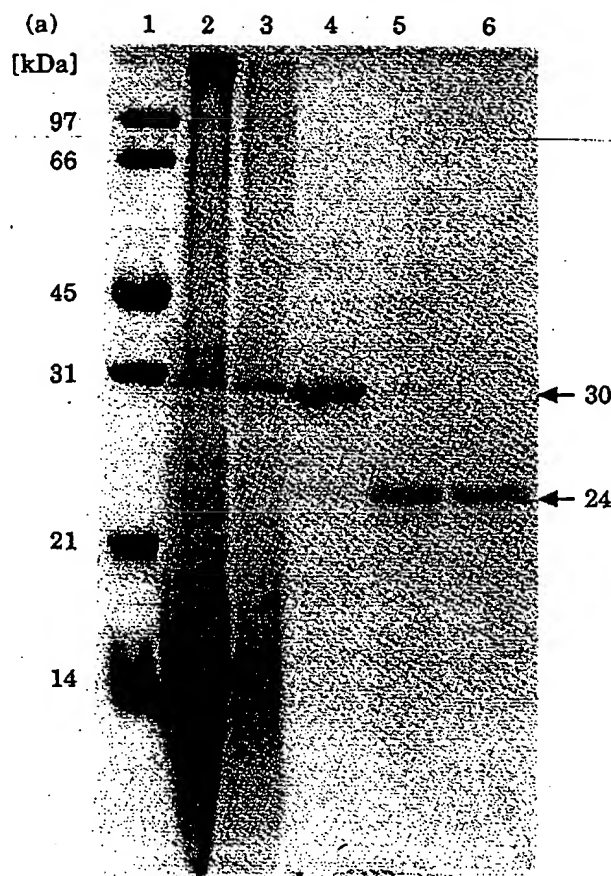


Fig. 2. SDS-PAGE analysis of the purification of the 30 kDa (unheated; 24 kDa heated) hemin-binding protein from *P. gingivalis* strain 381. Lane 1, low mol.wt. standards; lane 2, cell envelope fraction; lane 3, 1% CHAPS-soluble fraction; lane 4, isolation of 30 kDa protein; lane 5, 100°C, heated isolation 30 kDa protein; lane 6, purified 24 kDa protein.

Table 1 Cyanogen bromide fragmentation of 24 kDa hemin-binding protein from *Porphyromonas gingivalis* 381

Fragments	Amino acid sequence*
20 kDa	DQATSVPTDG(X)Y(X)TVD(X)KLGRITVK
17 kDa	GPDG(H)ZMEYEE
12 kDa	EYEEQGFSEVITGKKNAQGFAA(X)G(X)LEF(S)

*(X), unknown; (I), assume to be correct amino acid.

***N*-terminal sequence analysis**

The CNBr digestion of 24 kDa hemin-binding protein revealed at least three polypeptide bands (data not shown). Internal amino acid sequence analysis of one of these fragments (12 kDa) is seen in Table 1.

Discussion

Bacterial growth and membrane protein expression which are regulated by iron (hemin) have been reported in several microorganisms).⁷⁻¹¹ Many of these proteins have been implicated as functional components of iron (hemin) uptake systems

in these species. Although the requirement for hemin has been known for many years,¹² little is known about the mechanism(s) by which *P. gingivalis* binds and uptakes hemin into the cell. There have also been no reports of the purification and characterization of an actual hemin-binding protein from *P. gingivalis*.

Several Gram negative bacteria are known to utilize hemin as a sole source of iron. Hemin-binding proteins have been identified in several of these species, including *Shigella flexnerii*,¹³ *Bacteroides fragilis*,¹⁰ *Neisseria gonorrhoeae*,¹⁴ *Hemophilus influenzae*,¹⁵⁻¹⁷ *Treponema denticola*,⁷ and *P. gingivalis*.^{5,18} However, only few of these proteins have been purified and characterized. In the study presented here, we were able to establish that a 30 kDa (unheated) cell envelope associated protein from *P. gingivalis* strain 381 bound hemin and was stained with TMBZ. The expression of this protein appeared to be tightly regulated by the level of hemin(iron) in the growth medium.

Functionally, Omp 26 of Bramanti and Holt¹⁸ and Omp 32 of Smalley et al.⁵ appear similar with respect to hemin. Bramanti and Holt¹⁸ were unable to sequence Omp 26 because of N-terminal blockage, and Smalley et al.⁵ did not provide any sequence data for their Omp 32. Internal amino acid sequence analysis of the CNBr digested fragment and a search of GenBank for proteins with similar internal amino acid sequence to the 24 kDa protein revealed no significant similarities, and we consider the 30 kDa (heated 24 kDa) membrane protein to represent a newly described hemin binding protein from *P. gingivalis* strain 381. To our knowledge, the study described here is the first to identify, purify and biochemically characterize a hemin-binding protein from *P. gingivalis*. Work is in progress to further characterize the molecular structure of this protein.

Materials and methods

Bacterial strain and culture conditions. *P. gingivalis* 381 was grown anaerobically on the surface of enriched Trypticase soy agar, or in 2.1% (w/v) Mycoplasma broth base (BBL, Becton Dickinson, Cockeysville, MD) supplemented with 1 µg/ml menadione and 5 µg/ml hemin. Plate grown cultures were routinely incubated for 4 days and used as the inoculum for liquid growth. Liquid grown cells were incubated for approximately 24 h, equivalent to late exponential growth phase. For hemin restriction (i.e. hemin starvation), late exponential or early stationary phase cultures were grown with excess hemin (i.e. 7.7 µM hemin), and serially passaged at least 5 times as a 10% inoculum into hemin-free medium. Iron limitation was achieved by the addition of 100 to 400 µM of the iron-chelating compound, 2,2-bipyridyl (BPD., Sigma Chemical Co., St. Louis, MO), into liquid growth medium containing 7.7 µM hemin. All glassware was washed in chromic acid and rinsed in deionized water to remove contaminating iron and hemin. Culture purity was assessed by Gram staining and plating to solid medium.

Cell envelope preparation. Cells were harvested by centrifugation at 12,000 × g, for 20 min at 4°C, washed three times in cold phosphate-buffered saline (PBS, pH 7.2), and resuspended in PBS containing a protease inhibitor cocktail consisting of 2 mM each of phenylmethylsulfonyl fluoride (PMSF), benzamidine and Na-P-tosyl-L-lysine chloromethyl ketone (TLCK). Cell envelopes were prepared by French pressure cell disruption of whole cells in PBS (pH 7.2) by four 15,000 lb/in² disruption cycles. The cell envelopes were removed after low-speed (10,000 × g, 30 min) and high-speed (2,000,000 × g, 2 h) centrifugation. Protein concentration was determined using the bicinchoninic acid (BCA) assay of (Pierce, Lockford, IL).

Polyacrylamide gel electrophoresis. The discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Laemmli¹⁹ was employed for determination of protein distribution, and lithium dodecyl sulfate (LDS) PAGE was used

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for the TMBZ staining studies. 12% acrylamide separating gels were routinely used. All gels were run with a 4% acrylamide stacking gel in a vertical slab gel apparatus (Hoefer Scientific, San Francisco, CA). Proteins were visualized by Coomassie Brilliant Blue-R-250 stain (CBB). The hemin-associated peroxidase activity of the cell envelope protein was determined by tetramethylbenzidine (TMBZ, Sigma Chemical Co., St. Louis, MO) staining of LDS-PAGE gels. The TMBZ staining was carried out as described by Stugard *et al.*¹³

Purification of hemin-binding protein. Isolation of hemin-binding protein from *P. gingivalis* 381 cell envelope was accomplished by solubilization in the zwitterionic detergent 3 [(3-chloramidopropyl)-dimethyl-ammonio] 1 propane sulfonate (CHAPS; Pierce, Rockford, IL). Cell envelopes from *P. gingivalis* 381 (passage 5) were isolated as described above, and solubilized by the addition of CHAPS to a final concentration of 1% (v/v) and incubated at 37°C, 1 h. The resulting suspension was ultracentrifuged at 100,000 g for 1 h to pellet insoluble material, and the CHAPS-soluble fraction was either used immediately for the purification of hemin-binding protein or stored at -20°C until used. The hemin-binding protein referred to as 'unheated 30 kDa' (see Results) from *P. gingivalis* 381 was purified from the CHAPS-soluble membrane fraction of hemin-starved passage five cells by 1D-SDS-PAGE through a 12% gel employing a preparative comb with one reference well. The elution protocol of Hager and Burgess²⁰ was used. Purity of the isolated protein was confirmed by 1D SDS-PAGE.

Cyanogen bromide digestion and N-terminal sequencing. Initial experiments revealed that the unheated 30 kDa protein did not transfer well to a polyvinylidene difluoride (PVDF) membrane (Pro Blot, Applied Biosystems, Foster City, CA). Heating the 30 kDa protein at temperatures above 70°C resulted in the modification of the protein to a molecular weight of 24 kDa. In this heated, denatured condition the protein transferred quantitatively, and was used for N-terminal amino acid analysis. Attempts to directly sequence the N-terminus of the 24 kDa protein were unsuccessful because of a blocked N-terminus. Therefore, sequences were determined after cyanogen bromide (CNBr) digestion.

Cleavage by CNBr was carried out on the acetone precipitated 24 kDa protein. Several crystals of CNBr were added to 100 µl of 70% formic acid and swirled to dissolve. 50 µl of this solution was added to the acetone precipitated sample and allowed to proceed in the dark at room temperature, 16 h. The digest was dried under an N₂ stream and reduced to dryness in a SpeedVac SC 100 (Savant). The dried digest was dissolved in 1× treatment buffer for SDS-PAGE, heated at 100°C for 5 min, electrophoresed through an exponential gradient gel (7.5 to 20%), and electroblotted to a PVDF membrane at 100 mA, 4 h. After transfer, the ProBlot membrane was removed from the transblotting sandwich and rinsed with deionized water. Protein bands on the ProBlot membrane was visualized by Amido black staining. The protein bands were excised from the dried membrane, and its N-terminal amino acid sequence was determined with an Applied Biosystems (Foster City, CA) Model 477A gas-liquid phase sequencer coupled to an on line high-performance liquid chromatography model 120A analyzer.

This work was supported by USPHS grant DE-07627 from the National Institute of Dental Research.

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Instructio

Scope

Microbial Pathogenesis is the belief that biological processes are completely determined by the virulence factors that can now be used to control the T cell immune response. The biology of infection and pathogenesis of pathogens, including pathology, must be approached from a new perspective, namely a recognition that comparative biology is the whole rationale.

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Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in periodontal disease

EDITOR
Jørgen Slots

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Contributors

Sirkka Asikainen, DDS, PhD

Docent

Institute of Dentistry
University of Helsinki
Helsinki, Finland

Catherine Brissette, MS

Technician

University of Washington
Seattle, Washington, USA

Casey Chen, DDS, PhD

Associate Professor

Department of Periodontology
School of Dentistry
University of Southern California
Los Angeles, California, USA

Jeffrey L. Ebersole, PhD

Professor

Departments of Periodontics and Microbiology
University of Texas Health Science Center at
San Antonio
San Antonio, Texas, USA

Magda Feres, DDS, MS

Post Doctoral Research Fellow

Department of Oral Biology
Harvard School of Dental Medicine
and
Department of Periodontology
Forsyth Dental Center
Boston, Massachusetts, USA

Paula Fives-Taylor, PhD

Professor

Department of Microbiology & Molecular Genetics
University of Vermont
Burlington, Vermont, USA

Caroline Attardo Genco, PhD

Associate Professor

Departments of Medicine and Microbiology and
Immunology
Boston University School of Medicine
Boston, Massachusetts, USA

Saheer E. Gharbia, PhD

Lecturer in Microbiology and Immunology

Department of Life Sciences
University of East London
London, England, United Kingdom

Anne D. Haffajee, BDS

Senior Member of Staff

Department of Periodontology
Forsyth Dental Center
Boston, Massachusetts, USA

Stanley C. Holt, PhD

Professor

Department of Microbiology
University of Texas Health Science Center at
San Antonio
Graduate School of Biomedical Sciences
San Antonio, Texas, USA

Lakshmyya Kesavalu, DVM

Postdoctoral Fellow

Department of Microbiology
University of Texas Health Science Center,
San Antonio
San Antonio, Texas, USA

Denis F. Kinane, BDS, FDS, PhD

Professor

Departments of Periodontology and
Oral Immunology
Glasgow Dental Hospital and School
Glasgow, Scotland, United Kingdom

Donna Mager, DDS

Post Doctoral Research Fellow

Department of Oral Biology
Harvard School of Dental Medicine
and
Department of Periodontology
Forsyth Dental Center
Boston, Massachusetts, USA

Diane Hutchins Meyer, PhD
Research Assistant Professor
Department of Microbiology & Molecular Genetics
University of Vermont
Burlington, Vermont, USA

Keith Mintz, PhD
Research Associate
Department of Microbiology & Molecular Genetics
University of Vermont
Burlington, Vermont, USA

Kenneth T. Miyasaki, DDS, PhD
Associate Professor
Section of Oral Biology
UCLA School of Dentistry
Los Angeles, California, USA

John Mooney, MSc, PhD
Research Associate
Periodontal and Oral Immunology Research Group
Glasgow Dental Hospital and School
Glasgow, Scotland, United Kingdom

Frank C. Nichols, DDS, PhD
Professor
Department of Periodontology
University of Connecticut Health Center
School of Dental Medicine
Farmington, Connecticut, USA

Ingar Olsen, DDS, PhD
Professor
Department of Oral Biology
Dental Faculty
University of Oslo
Oslo, Norway

Haroun N. Shah, PhD, FRCPath
Head
Identification Services Unit
PHLS Central Public Health Laboratory
National Collection of Type Cultures
London, England, United Kingdom

Jørgen Slots, DDS, DMD, PhD, MS, MBA
Professor and Chairperson
Department of Periodontology
and
Associate Dean for Research
School of Dentistry
University of Southern California
Los Angeles, California, USA

Sigmund S. Socransky, DDS
Senior Member of Staff
Department of Periodontology
Forsyth Dental Center
Boston, Massachusetts, USA

Miriam Ting, DDS, MS
Resident of Advanced Periodontics
School of Dentistry
University of Southern California
Los Angeles, California, USA

Arie J. van Winkelhoff, PhD
Associate Professor
Department of Oral Microbiology
Academic Centre for Dentistry Amsterdam
Amsterdam, The Netherlands

Stephen Walker, PhD
Research Associate
Department of Microbiology
University of Texas Health Science Center
Graduate School of Biomedical Sciences
San Antonio, Texas, USA

Laurie Ann Ximenez-Fyvie, DDS
Post Doctoral Research Fellow
Department of Oral Biology
Harvard School of Dental Medicine
and
Department of Periodontology
Forsyth Dental Center
Boston, Massachusetts, USA

Homayoun H. Zadeh, DDS, PhD
Assistant Professor
Department of Periodontology
School of Dentistry
University of Southern California
Los Angeles, California, USA

Virulence factors of *Porphyromonas gingivalis*

STANLEY C. HOLT, LAKSHMYA KESAVALU, STEPHEN WALKER
& CAROLINE ATTARDO GENCO

"There are some bacteria that cause a disease, but there are some diseases that bring about a condition that is ideal for the growth of some bacteria."

Louis Pasteur

While classical studies on host-parasite interactions have considered a "virulence factor" to be a molecule(s) that exerts a detrimental effect (that is, toxin) on a host cell, recent observations have revealed that virulence factors are better described as molecules that result in the establishment and maintenance of a species associated with or within the confines of a host. Thus, while virulence factors are classically believed to harm the host, they can function in the establishment of a symbiotic or parasitic

relationship between the bacterial species and the host. In order for a potential virulence factor to exert its effects on a host, the bacterium must first find an appropriate ecological niche within that host (or site of activity), establish itself, and eventually grow and multiply. This establishment within a suitable site is essential for survival of the bacterium, and the production of maximum biological activity within the host environment. For example, most bacterial species that are able to invade a susceptible host do so by penetrating barrier membranes (that is, mucous membranes) to ultimately become associated with selected host cells (such as epithelial cells or fibroblasts). Adherence of a bacterium to its host or to other organisms resident in the host is an essential first step in colonization and pathogenicity. The invading bacterial species therefore must first breach the hosts external protective tissue barriers, then evade the constant action of cilia or the fluid movements of host cells, and find an appropriate ecological niche for colonization. Colonization of the host tissues is accomplished by a variety of putative virulence factors (Table 1), including fimbriae, lipoteichoic acids, lipopolysaccharides, exopolysaccharides, outer membrane proteins, and outer membrane vesicles.

Establishment of the bacterium within the host therefore is an essential first step in colonization. While several bacterial species are protected from host defenses by their intracellular location, most of the bacteria living associated with a host protect themselves by producing virulence factors that permit them to compete with the commensal host

Table 1. Virulence factors and host effectors produced by *Porphyromonas gingivalis*

Tissue destruction	Host evasion
Collagenase	Degradation of plasma protease inhibitors
Trypsin-like protease	
Gelatinase	
Aminopeptidase	Degradation of iron transport proteins
Phospholipase A	
Alkaline phosphatase	
Acid phosphatase	Inhibition of polymorphonuclear leukocytes
Chondroitin sulfatase	Chemotaxis inhibitors
Hyaluronidase	Decrease phagocytosis
Keratinase	Lysis and intracellular killing
Heparinase	
Nuclease	
Epithelioroxin	
Fibroblast growth inhibitors	Resistance to C' killing
Endotoxigenicity	C'3/C'5 degradation
Lipopolysaccharide-induced bone resorption	Immunoglobulin proteases
Volatile sulfur compounds	
Short-chain acid end products	Fibrinolysin
Indole	Superoxide dismutase
Ammonia	NADH oxidase
H ₂ S	Free-radical formation

microbiota by producing numerous antibacterial molecules. These molecules include bacteriocins and end-products of metabolism (organic acids, alcohols, inorganic bases, S-containing compounds, ammonia, etc.). A major protective mechanism available to many of the host-associated pathogenic bacterial species is the scavenging proteins, notable of which are the iron-binding proteins. Subsequent bacterial factors function to protect the now established bacterium from the numerous host defenses (especially serum factors including immunoglobulins and complement components). These bacterium-protective functions include the production of protective capsules (exopolysaccharides), which are both antiphagocytic as well as recognized to "mimic" host tissue and become immunologically "transparent". A variety of enzymes are also produced by the invading bacterium that are either essential for providing nutrients for bacterial metabolism or function to destroy host defense molecules (Table 1). The best example of these types of enzymes are produced by a variety of gram-negative bacteria and are referred to as cysteine proteinases, which are capable of degrading and inactivating several essential host-defense mechanisms. The O-antigen of the lipopolysaccharide (in gram-negative bacteria) as well as the capsular polysaccharide also function to protect the bacterium from phagocytic events by permitting complement fixation (required for effective phagocytosis) at sites distant from the colonization niche and are thus resistant to the lethal lytic effects of host serum proteins.

The production of selected enzymes and bacterial end-products provides the resident bacterium with the mechanisms(s) to survive and multiply in the confines of a host. The ability to utilize host metabolites and produce end-products that are noxious or even toxic to other bacterial species, and even to host cells, provides a uniqueness to the ecological niche that can result in the growth of a potentially virulent bacterial species and the production and expression of numerous proteins (that is, virulence factors) that are either harmful to the host or important growth factors for the bacterium.

Periodontal diseases

Periodontal diseases are a group of inflammatory diseases of the gingiva and the supporting structures of the periodontium. They are the most common of the oral inflammatory diseases and are described as the bacterially initiated conversion of a healthy gin-

gival region to one characterized by inflammation (gingivitis) and the destruction of the supporting structures of the teeth (periodontitis).

While a large number of different species have been recognized as members of the periodontal environment, it is now recognized that it is not these large numbers of bacteria (bacterial load) that result in the biological progression from health to periodontal disease (the nonspecific plaque hypothesis), but it appears to be the establishment and growth of a very few bacterial species among the 300 or more proposed different bacterial species resident in the subgingival niche that are periodontopathic (the specific plaque hypothesis). Among these putative periodontal pathogens are: *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Bacteroides forsythus*, *Campylobacter rectus*, *Prevotella intermedia*, the oral treponemes *Treponema denticola*, *Treponema pectinovorum*, *Treponema vincentii*, *Selenomonas sputigena*, *Eikenella corrodens* and *Porphyromonas gingivalis*. More than likely, no one of these species is capable of all of the destructive events involved in the inflammatory events and biological destruction of host tissue and bone observed in periodontal disease progression, but the process requires an integrated and orchestrated interaction of selected members of this periodontopathic ecology. Thus, periodontal disease is a multifactorial complex disease involving multiple bacterial species and host cell interactions, the combined effect of which is the destruction of soft tissue and bone. The individual proposed periodontopathic bacterial species elicit a large number of biological molecules that act on host tissue to destroy its integrity. In fact, while the potential participation of selected bacterial species is recognized to function as "putative periodontal pathogens", few of these have been shown to have a direct pathogenic effect on the host. One might postulate then that these bacterial species elicit a variety of putative "virulence factors" (few of which have been described *in vivo*), to which the host responds (bacterial insult) in ways that result in its own tissue destruction (Table 1). So, for example, while selected members of the periodontal environment might elicit potentially harmful virulence factors *in vitro*, these same factors in the confines of a host might not function at all or might only function indirectly on the host, eliciting host cell responses that result in the expression of host-derived hydrolytic enzymes and the activation of a variety of host-destructive cytokines. It is only recently that several research

groups have started to investigate the expression of these *in vitro* expressed virulence factors in the *in vivo* environment (147, 376).

P. gingivalis has long been considered an important member of the periodontopathic microbiota involved in periodontal disease progression and bone and tissue destruction (163, 379, 427). The organism is essentially absent during periodontal health, and during disease progression to periodontitis can reach a very significant percentage of the pathogenic microbiota. Return to oral health results in the absence or reduction in the number of *P. gingivalis* in the gingival environment. While other members of the periodontopathic niche ebb and flow as a function of oral health, *P. gingivalis*, because of its *in vitro* ability to produce significant numbers of potential virulence molecules, is considered an important pathogen in this progression from health to disease. Whether these virulence molecules, which have been described from bacteria grown *in vitro*, function in the confines of a host remains to be determined. It is more than likely correct that *P. gingivalis* along with other members of the host microbiota contributes to disease progression by interacting with selected host cells to produce host-cell destructive molecules. How these bacteria communicate with the host to trigger the conversion of a healthy oral tissue to a diseased one or whether these biological molecules actually function directly on host cells and tissues is unclear.

Description of the genus *Porphyromonas*

The species of the genus *Porphyromonas* (Table 2) have all been found associated with human and/or animal hosts. These species have been isolated from

the oral cavities of humans, dogs, cats, and nonhuman primates. More than likely, members of the genus are also found associated with a large number of other warm-blooded animals.

Members of the genus are 0.5–0.8 by 1.0–3.5 μm diameter and are obligately anaerobic, non-spore-forming, nonmotile rods. They have also been described as cocco-bacilli depending upon the stage of growth from which they are examined. Characteristic of the genus is the production of large amounts of cell-associated protoheme. When grown on complex carbohydrates (except the asaccharolytic *P. gingivalis*) and proteins, the major fermentation end-products are *n*-butyrate, propionate and acetate. These end-products account for much of the malodor associated with oral infections. Small amounts of *iso*-valerate *iso*-butyrate, succinate and phenylacetate are also produced. While several of the strains possess significant proteolytic activity (that is, *P. gingivalis* and *Porphyromonas macacae*), the other strains are relatively nonproteolytic.

P. gingivalis

Members of the *P. gingivalis* species are nonmotile, asaccharolytic, obligately anaerobic coccobacilli exhibiting smooth, raised colonies. When grown on a blood agar surface, the colonies are initially white to cream colored. With time (4–8 days) these colonies darken from their edge towards the center and a deep red to black color, which correlates with the concentration of protoheme. The species produce a large number of enzymes, proteins and end-products of their metabolism that are active against a broad spectrum of host proteins and provide mechanisms for evasion of host defenses (Table 1). These latter compounds include proteinase inhibitors, immunoglobulins, iron-containing proteins, bactericidal proteins, extracellular matrix proteins, and proteins intimately involved in phagocytic functions, such as complement fixation and coagulation (324). While many potentially unique enzymes (and metabolic end-products) are either cell-associated or excreted (see later), recent studies using molecular and immunological approaches have revealed that most of the enzymatic activity is due to the production of cysteine proteinases (that is gingipains; see later). Metabolically, the ability of *P. gingivalis* to secrete these cysteine proteinases in a host provides them with distinct advantages for its survival and growth, including the ability to use large host proteins for their growth and metabolism. Since these protein-

Table 2. Species of the genus *Porphyromonas*

Porphyromonas cangingivalis
Porphyromonas canoris
Porphyromonas cansulci
Porphyromonas catoniae
Porphyromonas circumdentaria
Porphyromonas crevioricanis
Porphyromonas endodontalis
Porphyromonas gingivalis
Porphyromonas gingivicanis
Porphyromonas levii
Porphyromonas macacae

Source: adapted from Collier L, Balow A, Sussman M, ed. Topley & Wilson's microbiology and microbial infections. 9th edn. Vol. 2. New York: Oxford University Press, 1998.

ases cleave both synthetic and host proteins after arginine and lysine residues, their growth is significantly enhanced in the presence of protein hydrolysates (trypticase, proteinase peptone and yeast extract).

In this chapter, we have elected to review the macromolecules associated with *P. gingivalis* that, *in vivo*, might function in the inflammatory and destructive events of periodontal disease. These include the capsule, outer membrane, its associated lipopolysaccharide, fimbriae, proteinases and selected enzymes. We also define the genetic tools that have been utilized to define the genes encoding these virulence factors and studies describing the regulation of these genes. In addition, the role of environmental cues as regulators of selected virulence factors and the interaction of *P. gingivalis* with host cells in various *in vitro* models are discussed.

Capsule

The presence of a capsule in *P. gingivalis* has been considered an important anti-phagocytic virulence factor by many investigators. Electron microscopic examination of several *P. gingivalis* strains by ruthenium red staining (for the presence of acidic mucopolysaccharides) and routine lead acetate staining has revealed the presence of an electron dense layer external to the outer membrane (Fig. 1). Some strains appear to be devoid of such a thick capsule and are either covered with a thin electron-dense layer, or are devoid of such a layer (143, 217, 302, 234, 248, 302). This electron dense-ruthenium red staining layer is the polysaccharide capsule.

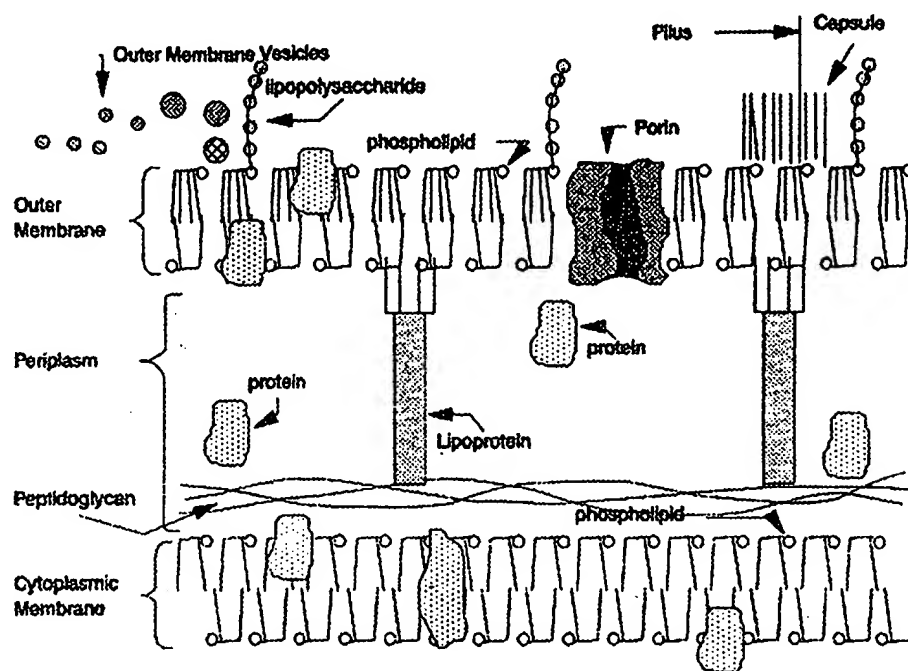
Chemical composition. The capsule from three *P. gingivalis* strains has been purified, composite sugars analyzed and shown to differ between strains (248, 302, 350). Mansheim & Kasper (248) determined that the capsule of *P. gingivalis* 381 contained galactose, glucose and glucosamine, whereas Okuda et al. (302) determined that the sugar composition of a similar strain was composed of rhamnose, glucose, galactose, mannose and methylpentose. Schifferle et al. (350) examined the capsule of *P. gingivalis* ATCC 53977 and found that it was immunochemically distinct from the lipopolysaccharide. Unlike the capsule of strain 381 and 382, *P. gingivalis* ATCC 53977 did not contain galactose and was rich in amino sugars. The capsule of the 53977 strain contained galactosamine, glucosamine, galactosaminuronic acid and glucose and displayed at least three serotypes of capsular antigens (428).



Fig. 1. Transmission electron micrograph of a thin section of *Porphyromonas gingivalis* strain W50. The cell was in the process of binary fission at the time of fixation. Note the thick, electron opaque capsule (C) that surrounds the asymmetric outer membrane (OM). Internal to the outer membrane is the thin peptidoglycan (Pg) in the periplasmic space (PS). The cytoplasm is surrounded by the unit cytoplasmic membrane (CM). The interior of the cell consists of a centrally placed nucleoid (N) surrounded by small, electron opaque ribosomes (R). Note the membrane fragments and membrane vesicles in the background (arrows).

Biological function. A strong relationship exists between the extent of *P. gingivalis* encapsulation and several important biological functions that could have a significant effect on its ability to function as an oral pathogen. The highly encapsulated *P. gingivalis* strains exhibit decreased autoagglutination, lower buoyant densities and were more hydrophilic than the less encapsulated strains (337, 404, 426). Increased encapsulation was also correlated with increased resistance to phagocytosis, serum resistance, and decreased induction of polymorphonuclear leukocyte chemiluminescence (48, 133, 404, 426, 428). The decreased tendency for the highly encapsulated strains to be phagocytized has been proposed to be due to the increased hydrophilicity of the strains and their decreased ability to activate the alternative complement pathway (352, 404). Schiffer-

Fig. 2. Diagrammatic representation of the gram-negative bacterial cell envelope. The asymmetric outer membrane contains the lipopolysaccharide, phospholipids, and numerous proteins. A major outer membrane protein, the porin, provides a mechanism for the selective transport of small-molecular-weight compounds into the cell. Attached to the surface of the outer membrane is the capsule, and pilus, or fimbria. Depending upon the growth environment, or the stage of growth, outer membrane vesicles of various sizes slough off the outer membrane. These vesicles contain lipopolysaccharide and outer membrane proteins. The outer membrane is attached to the cytoplasmic membrane by lipoprotein links through the periplasm, which houses the numerous hydrolytic, lipolytic and proteolytic enzymes found in gram-negative bacteria. Associ-



ated with the lipoprotein is the thin peptidoglycan, which provides the cell with its structural integrity.

le et al. (349) demonstrated that the *P. gingivalis* lipopolysaccharide (the O-antigenic polysaccharide) alone was responsible for activating the alternative complement pathway and not the capsule. These authors hypothesized that the thick capsule functioned to physically mask the lipopolysaccharide, and therefore the complement cascade could not be activated. The invading bacteria were therefore protected from opsonization and phagocytosis. Although some authors have suggested that the presence of a capsule increases the virulence of a particular *P. gingivalis* strain (48, 133, 337, 426), Sundqvist et al. (404) reported that only two of the nine encapsulated *P. gingivalis* strains were highly invasive in a mouse model. Therefore, the presence of a capsule in itself does not guarantee that a specific *P. gingivalis* strain will be virulent. The capsule also was found to interfere with the attachment of *P. gingivalis* to gingival epithelial cells.

P. gingivalis capsules conjugated to bovine serum albumin and to the *P. gingivalis* fimbriae protein (see later) have also been examined as vaccine candidates (51, 349). The capsule-bovine serum albumin conjugate, when used as an immunogen in mice, reduced the severity of a live *P. gingivalis* challenge, as judged by smaller secondary lesion formation and decreased weight loss compared with controls in a murine abscess model. However, this immunogen

did not prevent an invasive infection (349). The capsular polysaccharide-fimbrial protein conjugate vaccine was effective in preventing death after challenge with lethal doses of live *P. gingivalis*, and was also more effective than vaccines composed of fimbriae or capsule alone (51). The capsule antigen is therefore a very useful component of the cell surface providing protection from host defenses.

Outer membrane proteins

Structure of the gram-negative cell wall. Compared with the gram-positive bacterial cell wall, the "cell wall" of gram-negative bacteria is a complex multilayered structure (Fig. 2). Because of its multilayered, onion-skin construction, it is commonly referred to as the cell envelope. The cell envelope consists of the inner cytoplasmic membrane, a thin peptidoglycan, attached to which is asymmetrical outer membrane. The outer membrane contains the complex lipopolysaccharide, lipoproteins and peripheral and transport proteins. These latter proteins connect the outer membrane to the peptidoglycan and provide structural integrity to the cell envelope. Porin proteins provide a transport mechanism for the movement of selected proteins (approximately 600 Da) into and out of the cell. In most gram-negative bacteria the surface of the outer membrane is covered

by numerous thin short fimbriae, and if motile, by long, thick flagella. Lipopolysaccharides and hemagglutinins are intimately associated with the outer membrane. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins extracted from the outer membrane reveals that the outer membrane is composed of a complex of proteins ranging in molecular weight between 20 and more than 100 kDa.

Outer membrane of *P. gingivalis*. The majority of studies of the outer membrane proteins of *P. gingivalis* have relied upon the separation of the outer membrane by shearing, sonication of whole cells or separation of outer membrane proteins from outer membrane vesicles. Shearing and/or sonication of the cell envelope results in the separation of the outer membrane from the peptidoglycan and cytoplasmic membrane. Selected outer membrane proteins are then isolated from these fractions by detergent extraction and separated on columns and/or gels (70, 192, 440). SDS-PAGE analysis of these preparations revealed the outer membrane of *P. gingivalis* to contain at least 20 major proteins, ranging in size from approximately 20 to 90 kDa, many of which were heat modifiable (192). Radioiodination studies revealed that almost half of these outer membrane proteins (7 to 14) were surface exposed (192).

The virulence potential of several gram-negative bacterial outer membrane proteins has already been reported (*Vibrio*, *Mycoplasma* and *Neisseria*). Similar functional studies with the *P. gingivalis* outer membrane are only starting to appear. These *in vitro* studies have concentrated predominantly on the effects of "major outer membrane proteins" on epithelial cells, fibroblasts and a variety of bone cells. So, for example, Mihara & Holt (260–262) purified a 24-kDa protein from outer membrane vesicles of *P. gingivalis* strain W50 and observed the purified protein to be capable of stimulating thymidine incorporated human gingival fibroblasts. Because of its significant fibroblast-stimulating ability, these authors named this 24-kDa protein as a "fibroblast-activating factor". The protein was also capable of functioning as a cell proliferation factor for a number of eukaryotic cells and of stimulating bone resorption (262). In the calvarial bone assay (409), the 24-kDa outer membrane protein resulted in the release of significant amounts of ^{45}Ca from the rat calvaria. There was also the formation of large numbers of tartrate-resistant acid-phosphatase positive monocytes.

P. gingivalis also produces a 75-kDa major outer membrane protein that exists as a high molecular weight oligomer (392, 461). While the protein was often found to co-purify with the fimbriae, it was never found to be a part of the fimbrial structure. This outer membrane protein was also determined to be one of the immunodominant antigens of the organism (392, 461). While the exact function of this outer membrane protein has not been determined, Watanabe et al. (434) have found that the protein can stimulate polyclonal B-cell activation and can elicit interleukin-1 (IL-1) production from mouse peritoneal macrophages. The gene encoding the protein has been cloned and sequenced from two strains of *P. gingivalis* (294, 434). The sequence encoding the gene was highly conserved, and Southern blot analysis revealed that, in all of the *P. gingivalis* strains tested, only a single copy of the chromosomally-located gene was found. The protein is predicted to be produced with a signal sequence that is cleaved to generate the mature protein.

***P. gingivalis* outer membrane proteins and coaggregation.** The formation of dental plaque and the maintenance of its integrity relies upon the interaction of selected members of the microbiota. These aggregating or co-aggregating species form into complex microenvironments, or biofilms. *P. gingivalis* may play an important role in the formation and maintenance of the periodontal biofilm. The benchmark report of Gibbons & Nygaard (122) demonstrated that there were specific interactions between members of the oral microbiota and for the first time revealed that bacteria do attach to both hard and soft surfaces as well as to each other. With *P. gingivalis*, this interaction with selected gram-positive and gram-negative bacteria was mediated by specific outer membrane proteins in the whole cell-associated outer membrane or in the outer membrane vesicles. Coaggregation between *P. gingivalis* and *Actinomyces viscosus* was found to be important to the initial events in the formation of the subgingival biofilm (88, 127, 373). Kinder & Holt (201) also demonstrated that these co-aggregating pairs of resident gram-negative members of the periodontopathic microbiota also interacted with each other via specific adhesin-receptor molecules (Fig. 3). One of the *P. gingivalis* adhesins was identified by Hiratsuka et al. (158) to be a 40-kDa protein. While these investigators were able to clone the gene for the protein, they were unable to demonstrate coaggregation with the recombinant protein from *Escherichia coli*. However, affinity-purified antibody against the recombinant protein was able to signifi-

cantly inhibit the *P. gingivalis*-*A. viscosus* coaggregation. Coating of the *A. viscosus* with the recombinant protein also inhibited this coaggregation, indicating that this protein played an important role in the interaction of these two members of the plaque microbiota.

***P. gingivalis* and hemin.** Since *P. gingivalis* has an absolute growth requirement for hemin (iron), the presence of an active hemolysin associated with this bacterium was investigated (53). These investigators studied five *P. gingivalis* strains for their ability to lyse red blood cells, and all five strains produced a functional "hemolysin" associated with the outer membrane. While the hemolysin was produced throughout the growth cycle, significant amounts of the protein were formed during late exponential to stationary growth phase, with significant hemolytic activity being concentrated in the outer membrane

vesicles. Functionally, the concentration of the hemolysin in the vesicles could provide the important mechanism for the bacterium to compete effectively for hemin in the confines of the periodontal pocket. The large number of vesicles formed during growth could function to "attack" and hemolyze red blood cells during periods of active disease. Karunakaran et al. (188) cloned two *P. gingivalis* hemolysins, one 48 kDa and the other 18 kDa.

Bramanti & Holt (35) have provided some of the first data indicating that the expression of at least ten surface proteins with molecular weights between 26 and 80 kDa was observed in *P. gingivalis* W50 grown under hemin-deplete conditions. Growth of *P. gingivalis* strain W50 in the presence of normal to excess hemin (hemin replete; required for growth of *P. gingivalis*), resulted in the downregulation of these proteins. Growth under conditions in which hemin was a limiting growth factor (hemin deplete) resulted in the expression of at least 12 outer membrane proteins not found in the outer membrane of cells grown in hemin replete conditions. Two of these "hemin-regulated outer membrane proteins", at approximately 83 and 26 kDa, were recognized by SDS-PAGE analysis as major hemin-regulated *P. gingivalis* W50 outer membrane proteins. Bramanti & Holt (33) postulated that the hemin repression observed required the presence of protoporphyrin IX. Growth of the hemin-depleted cultures in normal or hemin-replete medium resulted in the rapid repression of the OMP26 (as well as the other hemin-regulated outer membrane proteins). However, OMP26 was not repressed in the presence of lactoferrin, transferrin, inorganic iron, zinc protoporphyrin IX or protoporphyrin IX. Radioiodination studies revealed that OMP26 was no longer accessible to iodine labeling after less than one minute of culture shift from hemin-deplete to hemin-replete culture (36). SDS-PAGE analysis revealed that OMP26 migrated with an apparent molecular weight of 39 kDa in its unheated closed molecular configuration. High-resolution immunogold electron microscopy confirmed the rapid migration of OMP39 into the outer membrane, where it was no longer surface accessible to both immunogold labeling and radioiodination (36). Therefore, OMP26 might function to bind hemin under hemin-depleted conditions and transport it across the outer membrane and into the cytoplasmic region where it would be used in a variety of anabolic reactions. Bramanti & Holt (34) also reported that hemin-starved cells bound much higher amounts of [⁵⁵Fe]-hemin than cells grown in heme-rich conditions. Purified monospecific polyclonal

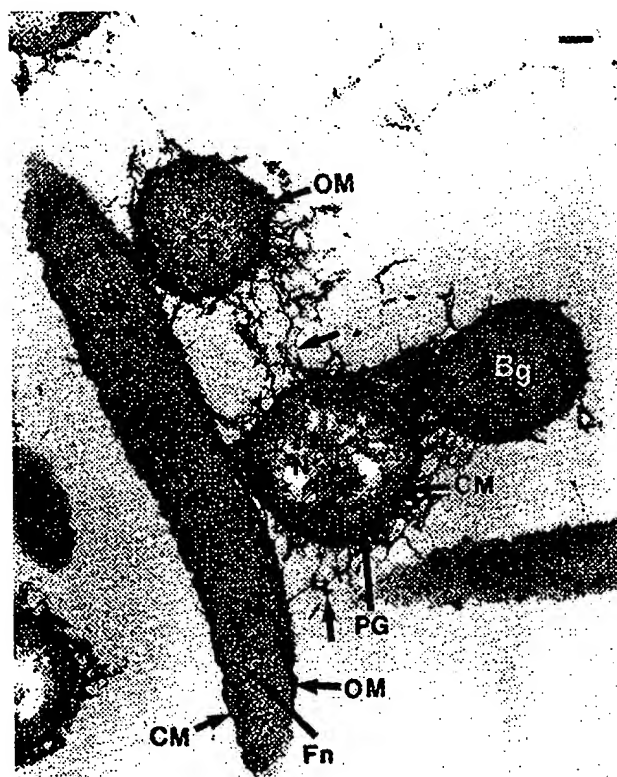


Fig. 3. Coaggregation between *Porphyromonas gingivalis* (Bg) and *Fusobacterium nucleatum* (Fn). The co-aggregation between the two strains is mediated by the thin, electron opaque fimbriae (arrows). CM=cytoplasmic membrane; N=nucleoid; OM=outer membrane; PG=peptidoglycan; R=ribosomes. Bar=0.1 μ m. Courtesy of S. Klinger-Haake.

anti-26 kDa antibody was capable of inhibiting hemin binding to hemin-starved cells. The binding of the hemin to the cells was also determined to be mediated by the protoporphyrin IX molecule since unlabeled hemin, protoporphyrin IX, zinc protoporphyrin and Congo red inhibited [^{55}Fe]-hemin binding but non-hemin iron sources such as transferrin and lactoferrin did not inhibit hemin binding. Direct binding of hemin to OMP26 was observed using a photoreactive cross-linking reagent. Recently, Kim et al. (200) isolated, purified and biochemically characterized a 30-kDa (heated=24 kDa) hemin-regulated hemin-binding protein from *P. gingivalis* strain 381. This protein was very similar to OMP26 from strain W50 and is more than likely its homologue in strain W50. *N*-terminal amino acid sequence analysis of the purified protein revealed no known homology with other hemin-binding classes of proteins.

Lipopolysaccharide

The outer membrane of gram-negative bacteria lies external to the peptidoglycan and is attached to it by selected lipoproteins (Fig. 2). These lipoproteins or murein lipoproteins attach by both covalent and non-covalent bonds to protein units within *P. gingivalis* and to the outer membrane by their lipid moieties. The outer membrane of gram-negative bacteria is asymmetric, the outer leaflet of which contains the lipopolysaccharide. The lipopolysaccharide is a very large molecule, with estimates ranging from 10 kDa and larger. Its amphipathic character is a result of one end of the molecule, the hydrophilic end consisting of the polysaccharide or *O*-specific (somatic) antigen, which is exposed to the environment on the exterior surface of the outer membrane, and the core region, buried within the outer leaflet which connects the *O*-antigen to the hydrophobic end of the molecule or lipid A (Fig. 2). This complex lipid is embedded in the lipid portion of the outer membrane leaflet.

The reader is referred to any basic textbook on bacterial structure and chemistry for an in-depth discussion of the chemistry and biological activity of this large and complex macromolecule.

Chemical composition. The lipopolysaccharide from several strains of *P. gingivalis* have been isolated, purified and chemically characterized (39, 40, 108, 183, 207, 210–212, 271, 288). While several initial studies indicated that these lipopolysaccharide molecules are different from those of the more classical enterobacterial lipopolysaccharides (*Salmonella* and *Escherichia*; Fig. 4), other studies re-

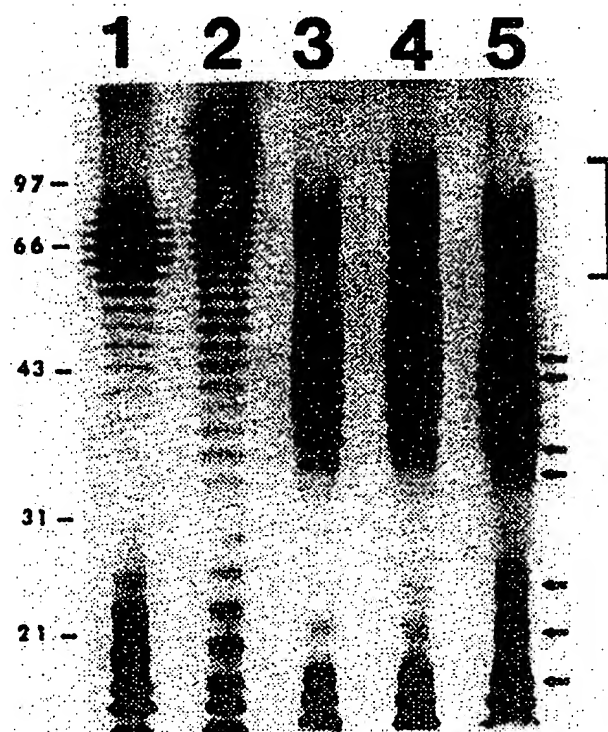


Fig. 4. 10% SDS-PAGE analysis of the lipopolysaccharides of *Porphyromonas gingivalis* strains W50, W83, ATCC 33277, *Escherichia coli* 0217:B8 and *Salmonella typhimurium*, S-type. Lane 1: *E. coli* (5 µg); lane 2: *S. typhimurium* (5 µg); lanes 3–5: *P. gingivalis* W50, W83 and ATCC 33277, respectively (15 µg each). Molecular weight are indicated on the left side of the gel. Arrows indicate differences in lipopolysaccharide unit migration for strain ATCC 33277 compared with W50 and W83. The *P. gingivalis* strains exhibited a smooth lipopolysaccharide chemistry similar to the enteric lipopolysaccharides.]=cluster of lipopolysaccharide subunits (58 to 100 kDa) that could be dissociated by deoxycholate-PAGE. Silver stained.

vealed a chemistry which was similar to that of the enterobacterial lipopolysaccharide (Table 3). For example, Nair et al. (271) and Koga et al. (207) reported that the isolated lipopolysaccharide from *P. gingivalis* strains lacked heptose, 2-keto-3-deoxyoctonate and β -hydroxy decanoic acid. While Bramanti et al. (39) and Johnne et al. (183) either found that their *P. gingivalis* lipopolysaccharide either lacked 2-keto-3-deoxyoctonate or only found small amounts, it is more than likely that the periodate and thiobarbituric acid assay used by these investigators was not capable of detecting the phosphorylated form of the sugar. Kumada (211) and Fujiwara et al. (108) determined that in *P. gingivalis* strain 381, the 2-keto-3-deoxyoctonate molecule was indeed phosphorylated at position C7 or C8 and thus would not have been detected by the classical method for 2-keto-3-deoxyoctonate identification.

Table 3. Chemical composition of lipopolysaccharide isolated from selected *Porphyromonas gingivalis* and *Escherichia coli* strains

Lipopolysaccharide	Sugar							Amino sugars	Percentage fatty acids (lipid A) (%)	Percentage of total phosphorous (%)
	Rhamnose	Fucose	Mannose	Galactose	Glucose	Heptose	2-keto-3-deoxy-octonate			
<i>P. gingivalis</i>										
381	++	-	+	+	++	-	-	++	18	1.5
W50	++		++	++	++			±	47.3	2.3
W83	++		+	++	++			±	47.7	2.3
ATCC 33277	++		++	++	++			±	57.5	1.9
<i>E. coli</i>										
K235	±	++	+	+	++	7	6	++	26	2.5
JN109									23.7	2.2

° ++=10-40%, +=1-10%, ±=<1%, - =not detected. Sources: modified from Hamada et al. (Adv Dent Res 1988; 2: 284), Bramanti et al. (Oral Microbiol Immunol 1989; 4: 183), Kiley & Holt (Infect Immun 1980; 30: 862) and Masoud & Holt (Eur J Biochem 1991; 200: 775-779).

In a more recent study, Kumada et al. (211) reported that the polysaccharide portion of the lipopolysaccharide of *P. gingivalis* strain 381 was attached to the 2-keto-3-deoxyoctonate molecule. While most of the reported studies of *P. gingivalis* lipopolysaccharide were not able to detect heptose, two studies, those of Bramanti et al. (39) and John et al. (183), were able to detect trace amounts of this sugar. However, it is generally believed that *P. gingivalis* does not contain significant quantities of heptose in its lipopolysaccharide core. The fatty acid composition of several *P. gingivalis* strains has also been examined (39, 108, 161). Holt & Bramanti (161) have summarized these results and describe a fatty acid-lipopolysaccharide composition rich in a C15:0 iso-branched chain fatty acid ranging from 6 to 48% of the total acids depending on the strain. Interestingly, the straight chain alkanes (C14, C18:0) were essentially absent from these lipopolysaccharides, being replaced with large amounts of hydroxy fatty acids (C3-OH-16:0, 3-OH-iC17). For the most part, these lipopolysaccharides contain fatty acids that are branched and longer than those found in enterobacterial lipopolysaccharide (see below).

Sugar analysis of the lipopolysaccharide from at least six different *P. gingivalis* strains indicates that the lipopolysaccharide contains neutral sugars similar to that reported for the enterobacterial lipopolysaccharide. Rhamnose, mannose, galactose and glucose comprised the major neutral sugars, with galactose and glucose comprising the major sugars. While glucosamine and galactosamine represent the amino sugars present in the molecule, SDS-PAGE analysis and the relatively high molar ratios of sugars to fatty acids leads one to propose the molecule to be "smooth" in character (39, 108, 183, 207, 271). SDS-PAGE-silver staining of the purified *P. gingivalis* lipo-

polysaccharide reveals a pattern of ladder-like bands typical of "smooth" lipopolysaccharides (39, 62, 70, 108, 281). At present little is known of the detailed chemical structure of the O-antigen and core regions of *P. gingivalis* lipopolysaccharide.

In contrast to the O-antigen, the lipid A region from *P. gingivalis* (strains 381, SU) lipopolysaccharide has been studied thoroughly (210, 288). The lipid A of *P. gingivalis* 381 consists of a glucosamine β -(1-6) disaccharide 1-monophosphate backbone in which the hydroxyl groups at 3-, 3', 4-, 4'- and 6'- are free (Fig. 5). At positions 2 and 2' the acyl groups 3-OH-15-methylhexadecanoic acid and 3-hexadecanoyloxy-15-methylhexadecanoic acid, respectively, are amide linked to the backbone. The lipid A of strain SU 63 is also composed of a glucosamine β -(1-6) disaccharide 1-monophosphate backbone; however, position 4' is partially substituted with phosphate. The 2 and 2' positions are acylated, via amino groups, with 3-hydroxy-15-methylhexadecanoic acid and 3-O-(hexadecanoyl)-15-methylhexadecanoic acid, respectively, and the 3 and 3' positions are esterified with 3-hydroxyhexadecanoic acid and 3-hydroxy-13-methyltetradecanoic acid, respectively. The groups present on position 3 and 3' are thought to be reversible and the predominant lipid A species lacks one of these two groups.

Biological properties. A large number of studies have established the immunobiological importance of the lipopolysaccharide of the gram-negative cell envelope (see, for example, 333, 338, 339, 406, 438). In comparison to the classical lipopolysaccharide of *Salmonella typhimurium* and *E. coli*, numerous studies have determined that the endotoxicity of the isolated and purified *P. gingivalis* lipopolysaccharide when assayed by the *Limulus* amebocyte lysate as-

say, Schwartzman hypersensitivity assay, lethality in galactosamine-sensitized mice and a pyrogenicity assay was significantly less endotoxic than enterobacterial lipopolysaccharide (108, 271, 405). In contrast, an early study by Koga et al. (207) reported that the isolated lipopolysaccharide from *P. gingivalis* strain 381 displayed endotoxic properties similar to that of the enteric lipopolysaccharides. Since the biological activity of the lipopolysaccharide molecule has been demonstrated to be a function of its purity, especially in its content of contaminating protein, it is possible that the *P. gingivalis* lipopolysaccharides that elicited endotoxicity contained some contaminating protein. It is also possible that there are differences in chemical composition of the lipopolysaccharides between the different *P. gingivalis* strains used; however, while the quantitative chemistry of the lipopolysaccharides from several *P. gingivalis* strains did vary, qualitatively, there were no differences in the overall structure of the molecule.

Chemical dissection of the lipopolysaccharide into its component parts (O-antigen, core, lipid A) has permitted the determination of the biologically active components of the parent molecule. Endotoxic activity is confined to the lipid A, while significant immunobiological activity is contained within the O-antigen (406). Importantly, the recent isolation of a chemically defined lipid A from *P. gingivalis* strain 381 has permitted the conclusive evaluation of the biological properties of the molecule without concern for contamination (289, 299, 412). The endotoxic activity of this chemically defined lipid A from *P. gingivalis* strain 381 was compared with that of a synthetic lipid A from *E. coli* type (compound 506) and *Salmonella minnesota* type (compound 516). Supporting the previous reported observations that the *P. gingivalis* lipopolysaccharide possessed low to negligible endotoxicity, the *P. gingivalis* lipid A was 1000-fold less effective in a *Limulus* amebocyte lysate test than compounds 506 or 516 and induced only slight pyrogenicity at a dose 1000 and 100 times greater than that required to show a marked response using compound 506 and 516, respectively. In addition, no localized Schwartzman reaction was observed with the *P. gingivalis* lipid A. LD₅₀ studies using galactosamine-sensitized mice revealed that the *P. gingivalis* lipid A was approximately 2500 times more endotoxic than that of compound 506 and 516.

While the *P. gingivalis* lipid A possessed low endotoxicity, the synthetic lipid A was biologically active as an adjuvant, being capable of activating polyclonal B cells and agglutinating rabbit erythrocytes. It was as potent as compound 506 in polyclonal B-

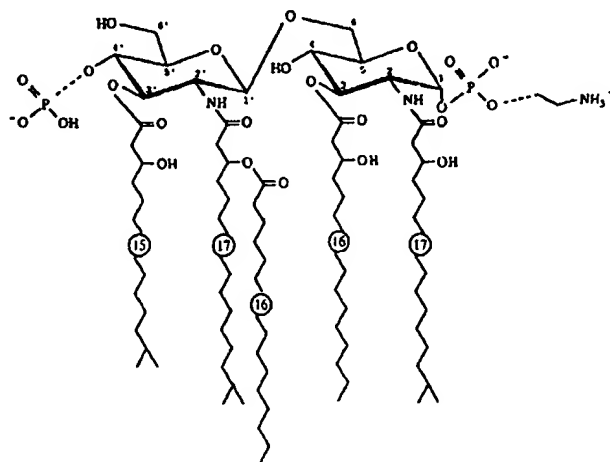


Fig. 5. Proposed chemical structure of the lipid A molecule from *Porphyromonas gingivalis*. The dotted bonds are indicative of minor substitutions. Positions 3 and 3' = esterified with (R) -3-OH-C_{16:0} and (R) -3-OH-C_{15:0}, respectively. Heterogeneity of fatty acid chain length and degree of acetylation occur in this lipid A. Source: Kumada et al. J Bacteriol 1995; 177: 2098-2106).

cell activation on BALB/c mouse splenocytes. Ogawa (289) also demonstrated that the lipid A as well as the intact lipopolysaccharide were capable of hemagglutination of rabbit erythrocytes. In contrast, Okuda & Kato (303) and Kirikae et al. (202) have reported that their lipopolysaccharide preparations from their *P. gingivalis* strains were inactive as hemagglutinins. Ogawa (289) also demonstrated that the defined lipid A, in comparison to compounds 506 and 516, also induced a comparable or stronger induction of IL-1 receptor antagonist, IL-6, IL-8, interferon- γ , and granulocyte-macrophage colony-stimulating factor in the culture supernatants of mononuclear cells or peripheral blood monocytes. However, this defined lipid A was a poor inducer of IL-1 β and tumor necrosis factor- α . The data reported by Ogawa et al. (299) appear to support the role of *P. gingivalis* lipid A as an IL-1 β antagonist in tissue cultures stimulated with compound 506 or *E. coli* lipopolysaccharide.

In addition to its ability to function as a chemokine inducer and antagonist, the *P. gingivalis* lipid A was also as effective (as compound 506) in protection of host cells from cytopathic effects. It was, for example, a strong inducer of natural killer cell activity in BALB/c spleen cells, had a greater ability to reduce cytopathology in the murine fibroblastic cell line L929 infected with vesicular stomatitis virus than the enterobacterial lipopolysaccharides and displayed significant antitumor properties.

Studies describing the biological activity of the de-

fined and isolated lipid A from *P. gingivalis* have also been compared with the original lipopolysaccharide samples from which the lipid A were derived (289). The intact native molecule was more toxic than the isolated lipid A as judged by lethality in galactosamine-sensitized mice and induction of clotting in the *Limulus* amebocyte lysate assay. The intact lipopolysaccharide was also more biologically active than the isolated lipid A as judged by its ability to stimulate polyclonal B-cell activation and interferon- γ . These latter results indicate that either other regions of the lipopolysaccharide molecule (in addition to the lipid A) exhibit biological activity or that the lipopolysaccharide was contaminated with other biologically active molecules (289, 299).

Tanamoto et al. (412) also examined the biological properties of the purified lipid A from *P. gingivalis* strain SU63 and compared it with the native molecule from the wild-type parent strain. Identical to the endotoxic activity of the other *P. gingivalis* lipopolysaccharides and isolated lipid A moieties, that isolated from strain SU63 was much less toxic than either the lipid A or the intact lipopolysaccharide from *S. minnesota* and compound 506 as assayed in galactosamine-sensitized C57BL/6 mice and in the *Limulus* amebocyte lysate test. Both the mitogenic response to the lipid A, as well as to the purified lipopolysaccharide of this strain in BALB/c spleen cells was similar and was 100 times lower than that induced by purified lipopolysaccharide from *S. minnesota*. This lipopolysaccharide and lipid A were also active in the induction of tumor necrosis factor- α and nitric oxide from peritoneal macrophages from C3H/HeN mice and the macrophage-like mouse cell line J774-1. However, the induction of these molecules was at least 100 times less than that induced by *S. minnesota* lipopolysaccharide. Identical to the macrophage-like mouse cell line J774-1, the human monocyte-macrophage cell line THP-1 was also stimulated to produce tumor necrosis factor- α by the *P. gingivalis* lipid A. Interestingly, it was more effective at low doses in stimulating the production of tumor necrosis factor- α than, for example, *S. typhimurium* lipopolysaccharide.

The studies by Ogawa et al. (299) and Tanamoto et al. (412) demonstrating that *P. gingivalis* lipopolysaccharide is a poor activator of IL-1 and tumor necrosis factor, as compared to enterobacterial lipopolysaccharide, are in contrast to other studies which demonstrated that *P. gingivalis* lipopolysaccharide stimulates equivalent or higher amounts of IL-1 or IL-1 β (6, 142, 207, 451) and tumor necrosis factor- α (6, 233) from human and murine cells. Bra-

manti et al. (39) and Sismey-Durrant & Hopps (374) also reported that *P. gingivalis* lipopolysaccharide was able to stimulate the production of prostaglandin E₂ from mouse macrophages, and rat perios-teal and human gingival fibroblasts. Similar results reported by Tamura et al. (411) and Takada et al. (407) have also demonstrated IL-1 β and IL-8 stimulation by *P. gingivalis* lipopolysaccharide in human gingival fibroblast cells. In identical cell culture systems, the enterobacterial lipopolysaccharide was not active in the induction of these cytokines. While there are reports that *P. gingivalis* lipopolysaccharide is capable of inducing IL-6 in human gingival fibroblasts (22), this induction is thought to be the result of an indirect effect of the lipopolysaccharide stimulating the production of IL-1 β (451). Yamaji et al. (447) have also demonstrated the induction of IL-6 and IL-8 in human periodontal ligament fibroblasts when exposed to *P. gingivalis* lipopolysaccharide. In this culture system, these investigators were also able to demonstrate that the induction of IL-8 by *P. gingivalis* lipopolysaccharide was significantly greater than that stimulated by *E. coli* lipopolysaccharide. Therefore, the preponderance of the evidence indicates that the *P. gingivalis* lipopolysaccharide, especially its lipid A, is capable of stimulating the host inflammatory response indirectly via the induction of host derived cytokine production.

Darveau et al. (66) have also investigated the ability of *P. gingivalis* lipopolysaccharide to stimulate the inflammatory response directly by its interaction with endothelial cells. They found that unlike *E. coli* lipopolysaccharide, *P. gingivalis* lipopolysaccharide did not stimulate the expression of E-selectin in human umbilical cord endothelial cells nor did it stimulate neutrophil adhesion to these cells. Employing an *in vivo* mouse model, Reife et al. (336) demonstrated that purified *P. gingivalis* lipopolysaccharide mediated a variety of inflammatory responses; however, it did not induce an inflammatory cellular infiltrate, the production of P- or E-selectin or the production of monocyte chemoattractant protein 1 or fibroblast-induced cytokine. In comparison, the lipopolysaccharide from *E. coli* was able to induce all of these events.

While the studies of Tanamoto et al. (412), Ogawa (289) and Ogawa et al. (299) have confirmed the biological properties of the *P. gingivalis* lipopolysaccharide to be significantly different from that of the enterobacterial lipopolysaccharide, especially with regard to its endotoxicity, these investigators, as well as Kumada et al. (210), and Ogawa (288) have reported that the *P. gingivalis* lipopolysaccharide does

possess some, but not all of the immunopharmaceutical effects of classical enteric lipopolysaccharide. These differences in immunopharmaceutical activity are thought to be due to differences in the chemical structure of *P. gingivalis* lipopolysaccharide compared with that of the enterobacterial molecule, specifically, the absence of the 4'-phosphate group and the location and nature of the fatty acids on the *P. gingivalis* lipid A (289, 299, 406, 412). The recent study by Shapira et al. (363), has provided strong evidence that there are indeed differences in the endotoxicity of the *P. gingivalis* lipopolysaccharides. These investigators examined several biological activities of *P. gingivalis* strains A7436 and W50, compared with *Salmonella typhosa*. Strain A7436 was isolated from a clinical case of human aggressive periodontitis, while the original of W50 is unclear. Exposure of human monocytes and mouse macrophages to the lipopolysaccharides of these two strains resulted in a different expression of inflammatory responses, with regard to tumor necrosis factor- α and nitric oxide. *S. typhosa* was a potent inducer of nitric oxide compared with the *P. gingivalis* strains. Macrophages stimulated with 1 μ g of the *S. typhosa* lipopolysaccharide per ml secreted 2 to 3 times more nitric oxide than those stimulated with 1 μ g of strain A7436 lipopolysaccharide. Interestingly, there were no differences in tumor necrosis factor- α secretion between the *S. typhosa* lipopolysaccharide and that from strain A7436. However, in contrast to the lipopolysaccharide from *S. typhosa* and *P. gingivalis* A7436, the W50 lipopolysaccharide was without nitric oxide- and tumor necrosis factor- α -producing activity. These results by Shapira et al. (363) demonstrate quite clearly that there are clear differences in the activities of the *P. gingivalis* lipopolysaccharide molecules and that these differences in biological activity could have a profound effect on host-bacterial interactions and virulence outcome. These studies are consistent with those of Collins et al. (56) and Reife et al. (336), which have indicated that, in fact, *P. gingivalis* strains are different in their ability to induce inflammatory responses in *in vitro* experiments. The recent report by Frolov et al. (101) confirmed and extended the original report by Shapira et al. (363) on the effects of *P. gingivalis* lipopolysaccharide on cultured macrophages. Mouse macrophages elicited by either thioglycollate or chronic exposure to *P. gingivalis* strain A7436 were examined for their ability to form nitric oxide and tumor necrosis factor- α . Purified *P. gingivalis* A7436 lipopolysaccharide was used to measure the formation of nitric oxide and tumor necrosis factor- α secretion, as well as the expression

of IL-2, 4 and 6, and the response of thioglycollate-stimulated macrophages to pretreatment of the cells with the *P. gingivalis* lipopolysaccharide. The thioglycollate-stimulated macrophages produced only low levels of nitric oxide, and essentially no tumor necrosis factor- α . In contrast, stimulation of the cells with the *P. gingivalis* lipopolysaccharide resulted in an 8-fold increase in the secretion of nitric oxide, with no increase in the levels of tumor necrosis factor- α . The levels of nitric oxide were also significantly higher from the *P. gingivalis*-elicited cells, compared to those stimulated with thioglycollate. Both *P. gingivalis*- and thioglycollate-elicited macrophages expressed m-RNA for IL-2, tumor necrosis factor- α and interferon- γ . Both IL-4 and IL-6 were not expressed in these cells in response to *P. gingivalis* or thioglycollate. Pretreatment of the thioglycollate-elicited cells with the *P. gingivalis* lipopolysaccharide followed by a secondary lipopolysaccharide challenge resulted in a downregulation of tumor necrosis factor- α and an upregulation of nitric oxide. Therefore, the macrophage might respond selectively to the inflammatory challenge, and these functional changes might play some role in the local inflammatory response elicited by *P. gingivalis*.

Interaction of lipopolysaccharide with host cells: molecular studies. The molecular basis of the interaction of the *P. gingivalis* lipopolysaccharide with eukaryotic cells is just beginning to be investigated. The majority of these studies have been carried out with the enterobacterial lipopolysaccharide, predominantly *E. coli* and *S. typhimurium* (58, 332, 414, 445, 462, 494). These enterobacterial lipopolysaccharides were very effective in activating both myeloid and nonmyeloid cells via an interaction of the lipopolysaccharide with an lipopolysaccharide-binding protein, the lipopolysaccharide-binding protein complex, and with membrane bound and soluble CD14 proteins, respectively. Shapira et al. (364) determined that identical to the *E. coli* lipopolysaccharide, the *P. gingivalis* lipopolysaccharide stimulates cytokine secretion in monocytes by binding to CD14 after it had interacted with soluble serum factors. In contrast to that reported for the enteric lipopolysaccharides, *P. gingivalis* lipopolysaccharide was not able to activate nonmyeloid cells, and was, for the most part, significantly less effective than the enteric lipopolysaccharide in activating myeloid cells (59, 66, 289, 412). It appears that this inability of *P. gingivalis* lipopolysaccharide to stimulate myeloid cells is due to its approximately 100-fold lower ability to bind lipopolysaccharide-binding protein (59)

compared with that observed for the *E. coli* lipopolysaccharide. Recently, Shapiro et al. (365) have also demonstrated that the mechanism by which soluble CD14 binds to lipopolysaccharide-lipopolysaccharide-binding protein complex is different in *P. gingivalis* than in *E. coli*. Therefore, the results of Shapiro et al. (365) and Cunningham et al. (59) may explain why *P. gingivalis* lipopolysaccharide is a poorer stimulator of myeloid cells compared with that of enteric lipopolysaccharide. However, it does not explain why nonmyeloid cells are non-responsive to the *P. gingivalis* lipopolysaccharide. Since *P. gingivalis* lipopolysaccharide-lipopolysaccharide-binding protein complex are capable of binding to soluble CD14 (59), some other factor(s) must be responsible for the inability of these complexes to stimulate nonmyeloid cells when they come in contact with lipopolysaccharide from *P. gingivalis*.

In summary, the lipopolysaccharide of *P. gingivalis*

is chemically different from that found in the well-studied and benchmark enteric lipopolysaccharide. These chemical and structural differences more than likely reflect the functional differences between the two molecules (59, 365) and may relate to their role in the pathogenesis of periodontal disease. The low biological activity of *P. gingivalis*, especially its very low endotoxicity, may reflect the organisms ability to colonize and grow in sterile tissue undetected by the host (66).

Bacterial fimbriae

Structure. A large number of bacteria, especially the gram-negative, host-associated species have associated with their surface numerous, thin, straight appendages. These structures, which were first reported on members of the *Enterobacteriaceae*, were originally referred to as pili (77) and were shown to be important in red blood cell agglutination. These pili are now more correctly referred to as fimbriae, which describes their hair-like thin stranded character. Two major classes of fimbriae have been described; those that are involved in interaction with other bacteria and mammalian cells (adhesins) and, in the adherence to soft and hard cell surfaces are referred to as type-specific fimbriae, and those involved in bacterial conjugation are referred to as F- or sex-pili. These latter fimbriae are much longer and more flexible than the type-specific fimbriae and function in DNA transfer between cells.

The fimbriae from both the nonoral bacteria as well as from the other gram-negative prokaryotes are of uniform size, being approximately 3 to 25 nm in diameter, and 3 to 25 μ m long. Some fimbriae have been observed to be up to 20 μ m in length. While their overall size remains relatively constant, their distribution over the bacterial surface does vary. Several bacterial species have been observed to have as few as 10 fimbriae per cell, while others have as many as 1000.

To date, at least 8 kinds of type-specific fimbriae have been identified on various *E. coli* isolates; mannose-specific fimbriae, or type 1 fimbriae, are found in most species of the enterobacteria and are the most common. The type-specific fimbriae have been found to play an important function in the interaction with specific host cells, as well as being important to, for example, the production and delivery of selected toxins, and as colonization antigens. Several recent reports have indicated that the fimbriae of *E. coli* might also be involved in motility ("twitching") and in chemotaxis.

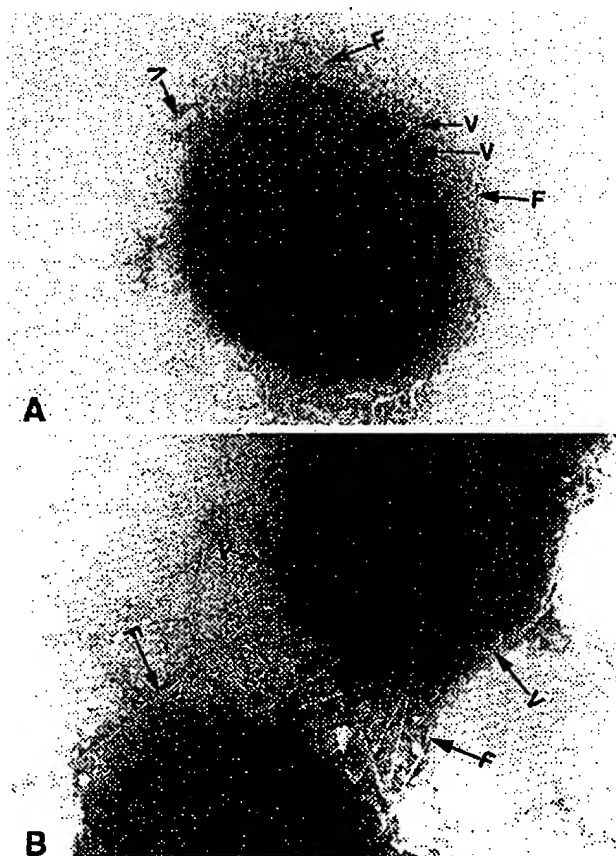


Fig. 6. A, B. Transmission electron photomicrographs of negatively stained cell surfaces of *Porphyromonas gingivalis* strain ATCC 33277. Numerous thin fibrils or fimbriae (F) emerge from the surface of the cells. The fimbriae are of uniform diameter; however, their length varies. Numerous outer membrane vesicles (V) are seen to be associated with either the surface of the outer membrane or free in the background.

P. gingivalis fimbriae. With only one or two exceptions, all of the *P. gingivalis* strains so far examined contain fimbriae arranged in a peritrichous fashion over the surface of the cell (Fig. 6) (143, 305). These structures have been isolated, purified and examined both chemically and structurally. SDS-solubilization to the monomeric subunits (fimbrillin) resulted in the formation of a polypeptide that migrates to a molecular weight of approximately 17 kDa (226, 391, 459, 460). The native fimbriae is comprised of at least 1000 protein subunits (fimbrillin subunits). Sex or "F" pili have not been described in any of the *P. gingivalis* strains so far studied.

The *P. gingivalis* fimbriae are not related to fimbriae produced by other black-pigmented *Bacteroides* species or to most of the other gram-negative prokaryotes. Different *P. gingivalis* strains produce fimbriae that are heterogeneous with respect to antigenicity, molecular weight of the fimbrillin subunit, N'-terminal sequence of the fimbrillin protein and DNA sequence (107, 226, 239, 295, 459, 460). The gene encoding the *P. gingivalis* *fimA* subunit has been cloned and characterized (72, 410). The *fimA* gene is resident on the chromosome as a single copy and all *P. gingivalis* strains so far examined contain the gene (72, 107, 368, 410, 368, 433).

Biological properties of *P. gingivalis* fimbriae. There is strong evidence that *P. gingivalis* fimbriae are responsible for binding the bacterium to host tissues and saliva-coated hydroxyapatite (see later). Purified fimbriae, recombinant fimbriae and synthetic peptides, based on the coding region of *fimA*, are effective in preventing the attachment of *P. gingivalis* cells to saliva-coated hydroxyapatite (227, 368). Based on experiments with recombinant fimbrillin, the salivary proteins statherin and the salivary proline-rich protein 1 from submandibular and sublingual-salivary components have been proposed as receptor proteins for fimbriae to bridge the fimbriae to the tooth surface (12).

Isogai et al. (176) showed that purified monoclonal antibodies to *P. gingivalis* 381 fimbriae could also inhibit the binding of the bacterium to human buccal epithelial cells in a dose-dependent fashion and that incubation of the bacteria with the antibody had no influence on the hemagglutination functions of the cells. In *P. gingivalis*, fimbriae do not bind to red blood cells, indicating that the fimbriae possess a host cell specificity. Watanabe et al. (435) in their studies of the interaction of *P. gingivalis* strains with human periodontal ligament fibroblasts, a human gingival fibroblast cell line (Gin-1), and the

human epithelial cell line Ca9-22, conclusively demonstrated that of the six *P. gingivalis* strains tested, three strains bound very well to the host cells, while three strains bound very poorly. Electron microscopy of the *P. gingivalis* strains that bound well to the epithelial host cells revealed abundant peritrichously arranged fimbriae 1–1.5 nm long and 0.5 nm wide on their surface, whereas the nonadherent strains had very few fimbriae. In addition to their sparse distribution over the bacterial surface, the fimbriae present in the poorly adhering strains were shorter than those found on the adherent cells. Antibody to the fimbriae of *P. gingivalis* strain 381 was found to inhibit adhesion of the bacterium to host cells, whereas an antibody to the major haemagglutinin of strain 381 did not inhibit binding, further confirming that binding of *P. gingivalis* to the epithelial cells, red blood cells and haemagglutination were separate processes. When tested for hemagglutination, the nonadherent *P. gingivalis* strains W83 and W50 had very similar activity as the adherent strain 381. *P. gingivalis* strains W50 and W83 were shown to possess few fimbriae but were highly virulent in a mouse model. The authors hypothesized that these strains resist phagocytosis in the mouse because they have few fimbriae to mediate attachment to the phagocytes. Duncan et al. (79) also tested a number of *P. gingivalis* strains for their ability to both bind and invade the human oral epithelial line, KB. Fimbriated *P. gingivalis* strain ATCC 33277 bound to KB cells as well as invading them. While these investigators did not demonstrate classical invasion, they were able to demonstrate that *P. gingivalis* strain ATCC 33277 was internalized into the cells. In contrast, strain W50 bound very poorly and did not invade KB epithelial cells. Approximately 10% of input strain ATCC 33277 cells bound to the KB cells, while <0.03% of strain W50 input cells bound to these epithelial cells. While it was not possible to determine whether the fimbriae were the mode of interaction with these eukaryotic cells, electron microscopy demonstrated that the ATCC 33277 cells were in tight association with the KB cell surface microvilli and that the bacteria tended to aggregate and form clumps on the monolayers.

Hamada et al. (136) used a genetic approach to assess the role of fimbriae in adhesion of *P. gingivalis* ATCC 33277 to human gingival fibroblasts and cell line Ca9-22 epithelial cells. They inactivated the *fimA* gene to create the mutant strain MPG1, which lacked the long fimbriae of the parent strain. Identical to some of the naturally occurring *P. gingivalis* nonadhering strains, the MPG1 mutant produced

small numbers of short fimbriae-like structures on their surface. These thin strands did not react to anti-fimbriae serum. Strain MPG1 also bound approximately three times less to the host cells than its fimbriated parent. Interestingly, mutant MPG1 displayed equivalent hemagglutination activity and hydrophobicity as the wild-type strain, indicative of another mechanism of interaction between red blood cells and *P. gingivalis*.

Recently, Weinberg et al. (437) investigated the role of fimbriae in attachment of *P. gingivalis* strains ATCC 33277 and 381 to gingival epithelial cells and the "invasion" of the attached bacteria into the interior of the fibroblasts. While both strains bound in comparable numbers to the primary gingival epithelial cells, strain ATCC 33277 was able to both adhere and invade host cells. These investigators also determined that a *fimA* knockout mutant of strain 381 (DPG3) bound only one half as well as the parent strain to host cells and was eight times lower in their ability to invade the cells. The ability of strain ATCC 33277 to invade the host cells was inhibited in a dose-dependent fashion by pretreatment of the cell monolayer with purified fimbriae. Weinberg et al. (437) also identified a 48-kDa epithelial cell surface protein that was bound strongly by ATCC 33277 and 381. However, all the strains also bound a 110-kDa host surface protein, while strain DPG3 also bound a 25-kDa host protein better than the other two strains. Weinberg et al. (437) hypothesize that the interaction of the fimbriae with the 48-kDa protein may be the first step in a signaling process that mediates uptake of the bacteria into the host cell. Unfortunately, they provided no supporting data for this hypothesis. In a recent report, Watanabe-Kato et al. (436), isolated 22 independent fimbriae-deficient mutants using transposon mutagenesis and immunological screening techniques. Southern hybridization analysis employing a probe for the *fimA* region, indicated that 9 mutants contained *fimA*, ORF1 (which encodes a 15-kDa protein), and the C'-terminal portion of ORF5 (which encodes a 63-kDa protein). The results identify genetic loci other than *fimA* that are potential regions for fimbriation in *P. gingivalis*. Njoroge et al. (285) have also investigated the role of the *P. gingivalis* fimbriae in adherence and invasion of KB epithelial cells. While both strains ATCC 33277 and 381 bound to epithelial cells with similar efficiencies, strain 381 was more efficient in invading this cell line. A third *P. gingivalis* strain A7436 was less effective than the other two strains in both invasion and adherence. Using *fimA* knockout mutants of strains 381, ATCC 33277, DPG3 and

MPG1, they determined that interruption of the fimbriae structural gene resulted in a nonadherent and noninvasive phenotype. The use of anti-fimbria serum also confirmed that the fimbriae were involved in both the binding and invasion of host cells.

Ogawa et al. (296) recently investigated the contribution of various regions of the fimbriae to binding to the human gingival fibroblast cell line, Gin-1. Purified, intact and radiolabeled fimbriae bound firmly to the surface of the fibroblasts. The synthetic peptides, when either added first to the fibroblast cells or concomitantly with the intact fimbriae, inhibited binding in a dose-dependent fashion. Peptides containing amino acids 1-20, 69-80, and 171-181 strongly inhibited binding of purified fimbriae, while two peptides, 282-301 and 302-321 were less inhibitory. Peptides 21-40, 81-101, 102-121, 142-161, 182-201, 202-221 and 242-261 did not inhibit the binding of the purified fimbriae to the Gin-1 cells. All of the inhibitory peptides, with the exception of 302-321 contained the sequence XLTX. Using Scatchard analysis, the authors determined that the Gin-1 cells had approximately 150 binding sites for the purified fimbriae and 18,440 sites for the synthetic peptide containing amino acid residues 171-181, and these binding sites were non-interacting-single class affinity sites. The dissociation constant for the fimbriae and peptide were approximately 15.9 pM and 29.2 nM, respectively.

Animal experiments have also been conducted that strongly implicate the *P. gingivalis* fimbriae as important virulence factors involved in tissue destruction (91, 92, 246). Immunization of gnotobiotic rats with *P. gingivalis* fimbriae resulted in the protection of the animals from periodontal damage when challenged with live *P. gingivalis* (92). Infection of gnotobiotic rats with a *fimA* knock-out strain of *P. gingivalis* resulted in a markedly reduced bone loss in comparison to animals infected with the wild-type strain.

The intact fimbriae, fimbrillin subunits or synthetic fimbrial peptides of *P. gingivalis* were also capable of eliciting several important host-associated biological responses that could result in harmful effects *in vivo*. All three fimbrial structures were capable of stimulating the production of fibroblast-derived thymocyte-activating factor from human gingival fibroblasts (159), IL-1, neutrophil chemotactic factor KC, and tumor necrosis factor- α from mouse peritoneal macrophages (140, 141, 268) and IL-6, IL-8 and tumor necrosis factor- α in human peripheral blood monocytes (293, 297). The fimbriae also functioned as adjuvants (293, 294), as well as being cap-

able of stimulating mitogenic and polyclonal B-cell activation in mouse splenocytes (293). An important characteristic of the *P. gingivalis* fimbriae was their chemotactic ability (290). This ability to sense host stimuli could have a significant effect on the formation of an inflammatory lesion as well as the progression of periodontal tissue and bone destruction (see below).

The analysis of human serum from both periodontally healthy individuals as well as from adult periodontitis patients revealed that diseased subjects had much higher antibody levels to fimbriae than did healthy subjects (291, 457). The fimbriae were also highly immunogenic, eliciting both an antibody and cell-mediated response in serum and saliva (138, 175, 216, 292, 294, 298).

The mechanism by which the *P. gingivalis* fimbriae interact with and induce a response in eukaryotic cells is under active investigation. While the details of the interactions are still unclear, it is known that after interaction of fimbriae with macrophages, there is the rapid induction of the neutrophil chemotactic factor, and protein kinase C (141, 269). Associated with this interaction is the formation of a 68-kDa phosphorylated protein within the confines of the macrophage, the induction being mediated by prior induction of protein kinase C.

Genetic examination of *P. gingivalis* fimbrial formation. In addition to the large number of studies on the structure, chemistry and function of the *P. gingivalis* fimbriae are the numerous studies on the genetics of fimbrial formation. Dickinson et al. (72) cloned and sequenced the gene encoding the fimbrillin subunit, *fimA*, from *P. gingivalis* 381. Export of the protein for the assembly of the fimbriae occurred through a leader sequence of 10 amino acids that, upon transport, were cleaved to produce the mature fimbrillin protein (72, 226). The gene encoded a protein of 35,924 Da, approximately 8000 Da smaller than the molecular weight of 43,000 Da predicted by SDS-PAGE analysis. In at least six *P. gingivalis* strains studied, the predicted molecular weights of the fimbrillin proteins varied from 37.5 to 38.2 kDa. The *fimA* genes from at least eight additional *P. gingivalis* strains have been cloned by polymerase chain reaction (PCR) and the proteins expressed in *E. coli* (107). Expression of the full length coding region in *E. coli* produced a protein approximately 2 kDa greater than that of the native fimbrillin isolated from whole cells of *P. gingivalis*, on the order of approximately 45 kDa. Thus, it is more than likely that the leader sequence is not processed in the heterologous host

and is accumulated in the *E. coli* recombinant as insoluble inclusion bodies. The *E. coli* recombinant fimbriae were, however, strongly recognized in Western blots using anti-*P. gingivalis* 381 fimbriae antibody for strains 381, ATCC 33277 and BH18/10. While the recombinant fimbriae from other *P. gingivalis* strains was also recognized by the *P. gingivalis* 381 antiserum, it was recognized to a lesser degree. Comparison of the fimbrillin DNA sequences demonstrated that strains 381, ATCC 33277 and BH18/10 contained *fimA* genes that were very similar, while the *fimA* of *P. gingivalis* strains HW24D1, OMZ314 and OMZ409 were similar to each other but significantly different from *P. gingivalis* strains 381, ATCC 33277 and BH18/10. *P. gingivalis* strains ATCC 49417 and 6/26 had similarities with strains HW24D1, OMZ314 and OMZ409; however, they were too divergent to be grouped with those strains. Analysis of the DNA sequence of strain HG 504 revealed that its *fimA* sequence was significantly different from the other *P. gingivalis* strains examined and might be the progeny of a completely separate ancestor. While the *fimA* gene sequences were found to be heterogeneous between the various species (see above), the 5' regions were very similar to each other. For example, all of the *P. gingivalis* strains examined, with the exception of strain HG 564, contained an identical leader sequence and also contained an identical putative -35 and -10 sequences. Both of these sequences were close to the *E. coli* consensus sequence, spaced 17 bp apart and utilized GTG as a start codon that was 15 bp upstream from a possible Shine-Dalgarno sequence.

The similarity of the *fimA* locus amongst the various *P. gingivalis* strains was examined by Southern blotting and restriction fragment-length polymorphism analysis. By Southern blotting, the *fimA* gene was found to be resident on the chromosome as a single copy gene in all *P. gingivalis* strains examined. The afimbriate *P. gingivalis* strains, and the other black-pigmented *Bacteroides* afimbriate species did not contain the *fimA* gene (72, 239, 410).

Restriction fragment-length polymorphism analysis has also been employed to study fimbrial polymorphisms in 39 *P. gingivalis* strains. A *P. gingivalis* strain 381 probe containing the *fimA* coding region was used, and the resulting restriction fragment-length polymorphisms revealed the presence of at least 9 groups. Group VIII (containing strains W50, W12, HG 564, AJW 5, and 9-14K-1) hybridized very weakly, while group IX (containing strain TG1, isolated from a sheep) did not hybridize at all to the probe, indicating little homology with *fimA* from

strain 381. However, the group VIII strains did hybridize well to probes containing DNA flanking the structural *fimA* gene, indicating that the *fimA* gene is located in the same region of the chromosome in the different strains. Thus, it is apparent from these studies that considerable heterogeneity in *fimA* exists among the various *P. gingivalis* strains and that, since only one *fimA* gene copy is found on the chromosome, the variation in the fimbrillin gene is most likely due to mutational events and/or genetic exchange between strains and not due to antigenic variation of the fimbriae within the strain. To date, no fimbrillin phase variation has been identified within a *P. gingivalis* strain.

Environmental effects on *P. gingivalis* fimbria formation. In addition to the genetic characterization of the *P. gingivalis* fimbriae, several investigators have also studied the influence of several environmental factors on the production of *P. gingivalis* fimbriae. These environmental effects included temperature, pH, hemin limitation, serum, saliva, osmotic effects and the effect of Ca^{++} -limitation. Amano et al. (9) employed Western blotting with anti-fimbriae serum and Northern blotting to study the effect of temperature on fimbrial formation. Growth of *P. gingivalis* strain 2561 at 39°C decreased the expression of fimbrial protein and mRNA by at least 50% in comparison to *P. gingivalis* 2561 grown at 37°C. Xie et al. (446) also studied the effect of temperature on fimbrial expression. However, these investigators used a genetic approach in their studies. They constructed a strain of *P. gingivalis* ATCC 33277 carrying a chromosomal transcriptional fusion of the 5' region of *fimA* and a promoterless *lacZ* gene. Using β -galactosidase activity as a measure of *fimA* transcription, they determined that an 11-fold increase in transcription of the fimbrillin gene protein occurred when the construct was grown at 34°C instead of 39°C.

Hemin limitation and growth in the presence of 1% serum or saliva reduced transcription of the fimbrial gene by 50% (9). However, changes in culture pH, Ca^{++} -ion concentration or osmolarity had no effect on the formation or integrity of the mature fimbriae. The decrease in β -galactosidase activity resulted in decreased fimbriation, and when the mutant was grown at low temperature, it displayed an increased ability to bind to *S. gordonii*, as well as to invade primary gingival epithelial cells; functions which are normally mediated by functional fimbriae. Since the α -amylase of *Streptococcus gordonii* has also been demonstrated to be involved in its interac-

tion with *P. gingivalis* and since this gram-positive streptococcus is devoid of fimbriae, it is possible that other mechanisms exist between gram-positive and gram-negative interaction.

Characterization of minor fimbriae species from *P. gingivalis* strains. Fimbriae produced by other bacteria, including *E. coli*, often contain minor proteins in addition to fimbrillin (the subunit that forms the bulk of the fimbria structure). Yoshimura et al. (458) cloned a 10.4-kb fragment of chromosomal DNA at the *fimA* locus of *P. gingivalis* 381 in order to identify the genes that might be involved in fimbrial morphogenesis. They identified three genes flanking *fimA* that were transcribed in the same direction as the fimbrillin gene. The gene encoding a 63-kDa protein was located upstream, and genes encoding 50- and 80-kDa proteins were found downstream of *fimA*. The proteins, or truncated versions, were expressed in *E. coli*, using a T7 RNA polymerase-promoter-expression system, purified and antibody raised against them. When SDS-PAGE gels were overloaded with purified fimbriae, five minor bands as well as the major band for fimbrillin were identified. Western blotting demonstrated that antiserum raised against the recombinant 80- and 50-kDa proteins reacted specifically with two of the minor fimbrial bands that had the same molecular weight as the recombinant proteins. Antiserum to the 63-kDa recombinant protein did not react with any proteins on the Western blot, suggesting that two of the recombinant proteins represent minor fimbria-associated proteins. The role of these minor fimbria-associated proteins in fimbrial function remains unclear. For example, Sharma et al. (368) demonstrated that the purified recombinant fimbrillin monomer (r-fim 10-337) was capable of inhibiting *P. gingivalis* whole cells from binding to saliva-coated hydroxyapatite. These results, as well as those obtained using synthetic fimbrillin peptides (227), suggest that no other protein but fimbrillin is required to mediate attachment of *P. gingivalis* to saliva-coated hydroxyapatite.

Construction of a *fimA* deletion mutant MPG1 of *P. gingivalis* ATCC 33277 produced a strain devoid of the classical, long thin fimbriae seen in the parent strain (136). However, electron microscopic examination of the mutant strain revealed that, while the long, thin, classical fimbriae were absent, the surface of these cells was sparsely covered with short fimbria-like structures, which when reacted with anti-fimbrial antibody were unreactive. Hamada et al. (135) isolated and characterized these "minor fim-

briae" from the mutant strain, and they were found to have a higher estimated molecular weight than the major fimbrillin: 67 kDa and 41 kDa, respectively. Amino acid compositional analysis of the two fimbriae showed that they contained similar amino acids and were rich in the hydrophobic amino acids. The two fimbriae were also antigenically distinct from one another. Antiserum against the 67-kDa fimbriae recognized the short (0.1–0.5 mm) fimbriae found on the mutant, while the wild-type strains did not react with the long (0.5–1.0 mm) fimbriae. While the wild-type fimbriae were involved in both adherence to epithelial cells and fibroblasts, as well as initiating hemagglutination reactions, these minor fimbriae did not possess any hemagglutinating activity. *P. gingivalis* ATCC 53978 (W50) is a poorly fimbriated strain that possesses a *fimA* gene that is thought to be non-functional due to a mutation in the initiation codon (137). Sojar et al. (390) purified the fimbriae from this strain ATCC 53978 and determined that the protein had a molecular weight of approximately 43 kDa, which appears to be more characteristic of the minor fimbriae. The oligomer was maintained even when heated at 100°C in 2% SDS. Conversion to its monomeric form occurred in the presence of reducing agents, at room temperature. The fimbrillin monomer from strain ATCC 53978 reacted with antiserum raised against peptides I and J of the fimbriae from strain 2561 in Western blotting experiments. The role of these minor fimbriae in *P. gingivalis*-mediated pathogenesis is not known.

Expression of *P. gingivalis* fimbrillin on *S. gordonii*.

Several very interesting studies have examined the initial bacterial colonization of a tooth surface and found that these early colonization events are mediated by specific members of the oral streptococci: *Streptococcus sanguis* and *S. gordonii*. These initial plaque colonizers attach to both saliva-coated hydroxyapatite surfaces and to other bacteria, including *P. gingivalis*. Recombinant strains of *S. gordonii* that expressed either the C-terminal or N-terminal portion of the *P. gingivalis* *fimA* gene were constructed. In frame fusions of DNA encoding amino acids 55–145 or 233–322 of *fimA* and the *Streptococcus* M6 protein gene were integrated into the chromosome of *S. gordonii* strain GP251. Analysis of the recombinant *S. gordonii* strains by Western blotting determined that a chimeric protein of 45 kDa was produced that reacted with anti-fimbrillin serum as well as a monoclonal antibody raised against the M6 protein. The fimbrial epitopes were

exposed on the surface of the *S. gordonii* strains, as judged by immunofluorescence microscopy and cell surface ELISA. Since the *fimA* gene of *P. gingivalis* has been shown to be involved in adherence to other bacteria, as well as to saliva-coated surfaces, the *S. gordonii* recombinant strains carrying *fimA* epitopes bound better than control *S. gordonii* to saliva-coated hydroxyapatite beads. In addition to greater binding efficiency, the recombinant constructs containing the N-terminal portion of the fimbriae bound better than those mutant strains expressing the C-terminal portion. The recombinant strains expressing the N-terminal sequence were also better than control *S. gordonii* at inhibiting the binding of *P. gingivalis* to saliva-coated hydroxyapatite. These recombinant *S. gordonii* strains (expressing C- and N-terminal ends of the *fimA* product) were also capable of stimulating antibody production in rabbits that recognized *P. gingivalis* fimbriae by Western blot.

Using a similar approach, Sharma et al. (367) expressed the N-terminal and C-terminal domains of *P. gingivalis* 381 fimbriae as a fusion protein with the N-terminal domain of the *S. gordonii* M6 protein. A transitional stop signal was placed at the 3' end of the fimbriae protein in order to prevent translation of the M6 C-terminal anchor domain. As a result, the fusion protein was not tethered to the surface of *S. gordonii* and was secreted into the external milieu. This recombinant *S. gordonii* strain produced a 28-kDa protein that reacted with anti-fimbrillin and anti-M6 serum. The strain producing the chimeric protein containing the C-terminal portion of the fimbrillin secreted approximately four times more recombinant protein than the strain containing the N-terminal portion of the fimbrillin. Sharma et al. (366, 367) propose to use these constructs in animal model experiments to attempt to interfere with colonization. It will be interesting to determine whether these constructs (the *S. gordonii* fimbria-expressing strains) prevent colonization by *P. gingivalis*. It is possible the constructs will stimulate an immune response to the fimbriae and physically block the adhesion of the pathogen by competition with soluble-recombinant fimbriae.

Non-fimbrial proteins. Recently, the role of several other surface-associated, non-fimbrial proteins from *P. gingivalis* has been investigated with regard to their interaction with host cells. In addition to their involvement in interaction between *P. gingivalis* and host cells, these non-fimbrial surface proteins also regulate the expression of the fimbriae. Kotani et al.

(208), Tokuda et al. (416) and Nakayama et al. (280) examined the surface of *P. gingivalis* strain 381 for molecules other than the fimbriae as potential adhesins. Several such proteins were identified and subsequently isolated and purified. These non-fimbrial components were found to be pure proteins, and biochemical characterization revealed them to be identical to the arginine-specific cysteine proteinases (see later). *N*-terminal amino acid sequencing of these proteins revealed that with the exception of residue 8, they were identical in their first 20 amino acids to Arg-gingipain. The proteinase was active against collagen type 1, fibronectin and bovine serum albumin; however, it demonstrated no activity against purified *P. gingivalis* fimbriae. In fact, the binding of *P. gingivalis* fimbriae to human gingival fibroblasts was inhibited by collagen, laminin and fibronectin. Exposure of fibronectin, collagen or the fibroblast cells to *P. gingivalis* cysteine proteinase digestion resulted in a significant enhancement of binding of the fimbriae to these surfaces. The presence of an L-arginine residue inhibited the binding of the purified fimbriae to collagen, and therefore exposure of host cells to proteinase treatment might "uncover" L-arginine residues, exposing them on the cell surface, and thus enhancing the binding of the fimbriae to the host cell. To test the hypothesis that these arginine-specific (cysteine) proteinases were involved in the interaction of *P. gingivalis* with host cells, Kotani et al. (208), Tokuda et al. (416), and Nakayama et al. (280) covalently bound various dipeptides to Sepharose beads and determined the ability of the purified fimbriae to bind to them. Only beads containing glycyl-arginine were capable of binding the fimbriae. These results are in contrast to studies reported by Watanabe et al. (435) in which L-arginine had no effect on the binding of *P. gingivalis* 381 to host cells.

Nakayama et al. (280) and Tokuda et al. (416) also employed a genetic approach to determine the role of the arginine-specific cysteine proteinases in host cell binding. Using an *rgpA* and *rgpB* knockout strain, *P. gingivalis* ATCC 33277 (KDP112), these investigators noted that the absence of RGP activity resulted in a non-fimbriated phenotype. Mutant strains with only one of the proteinase genes deleted produced decreased quantities of proteinase activity in comparison to the parent strain while still displaying wild-type fimbriation. Importantly, revertants of strain KDP112 that were wild-type for one of the two proteinase genes also produced normal fimbriae as judged by electron microscopy. Western blotting with anti-fimbriae serum of the various

strains indicated that the parent, as well as single-gene mutants, produced a single reactive band at 43 kDa corresponding to the processed-mature fimbrillin. The non-fimbriated double mutant showed two faint immunoreactive bands at 45 and 43 kDa, corresponding to both the non-processed and processed fimbrillin, respectively. Since *P. gingivalis* culture supernatants containing RGP residues can convert pre-fimbrillin produced in *E. coli* to the mature molecular weight molecule, and the predicted amino acid sequence of the pre-fimbrillin is capable of being cleaved into the mature protein by RGP, it is more than likely that RGP plays an important role in the maturation of fimbriae (308).

Tokuda et al. (416) and Kuramitsu et al. (214) also used molecular genetic approaches to determine the role of Arg-gingipain in interaction with KB cells (epithelial cells). They constructed a *P. gingivalis* 381 *rgp* knockout strain (G-102) and examined the interaction of this knockout mutant with KB epithelial cells. While strain G-102 bound to the KB cells, it did so with a 50% less efficiency compared to its wild-type parent. However, it did bind better than the *fimA* mutant from strain DPG3 (see below). Strain G-102 also displayed a significantly lower binding to fibronectin and laminin, was defective in its co-aggregation with *S. gordonii* or *A. viscosus* and displayed minimal autoaggregation compared to the parent strain. Revertants of the proteinase mutant (strain G-102) showed a wild-type phenotype, indicating that the site directed mutation in the proteinase gene was responsible for the mutant phenotype. Electron microscopic analysis of strain G-102 indicated that it lacked fimbriae but did possess low-density fibrils associated with the outer membrane, which appeared to be more "fragile" than the wild-type parent strain. Western blotting using anti-fimbria serum indicated that the mutant produced significantly reduced amounts of fimbrillin than the parent strain, but the fimbrillin produced was processed to the correct functional molecular weight. Concomitant with the reduced amount of fimbrillin produced was the demonstration by Northern blot analysis that the mutant produced greatly reduced fimbrillin mRNA, indicating that fimbrillin inhibition occurred at the level of transcription. Thus in *P. gingivalis* 381 and strain ATCC 33277, fimbria expression is influenced by the presence of a cysteine proteinase. Whether this surface-associated molecule itself is a major participant in interaction of *P. gingivalis* with eukaryotic cells is still equivocal, since it is not clear whether it is the mutation in gingipain or fimbriae that effects the association with the host cells.

Du et al. (76) recently reported a series of interesting experiments that employed an immunological approach to define *P. gingivalis* ATCC 33277 cell surface molecules which mediate attachment to membranes from SK-MES (ATCC HTB 58) epithelial cells. A ligand receptor interaction between the epithelial cell membrane and cell surface extracts of *P. gingivalis* was identified by crossed immunoaffinity electrophoresis methods. An immunoprecipitate band representing an interaction of a *P. gingivalis* surface antigen and a receptor on the epithelial cell surface was identified and used to make monoclonal antibodies. The purified monoclonal antibodies were capable of inhibiting bacterial adhesion to the epithelial cells, and Western blotting determined that each antibody recognized a number of *P. gingivalis* antigens, including the fimbriae.

In summary, the *P. gingivalis* fimbriae function similar to the fimbriae of other gram-negative bacteria. They are constructed of fimbrillin subunits, which form a mature oligomeric protein of 67 kDa. The *P. gingivalis* fimbriae that have been studied are involved in the interaction of the bacterium with both hard saliva-coated hydroxyapatite surfaces and with the surface of mammalian cells, such as epithelial cells and fibroblasts. Several animal studies indicate that rats immunized with purified *P. gingivalis* fimbriae protect the animals from live *P. gingivalis* challenge and associated bone and tissue destruction. Several very interesting studies of non-fimbrial *P. gingivalis* surface proteins reveals that the surface proteinase, cysteine proteinase, while itself a putative adhesin, might also function in the regulation and expression of the *P. gingivalis* fimbriae.

Proteinases

One of the potentially significant virulence characteristics of *P. gingivalis* is the large number of hydrolytic, proteolytic and lipolytic enzymes that are produced by essentially all of the known strains. Many of these enzymes are either exposed at the surface (in the outer membrane) of the bacterium where they are able to come into contact with host cells and tissues, within the periplasmic space capable of being transported to the cell surface, and in outer membrane vesicles, which are sloughed from the outer membrane during growth. Several of these *P. gingivalis*-associated proteases (proteinases capable of hydrolyzing peptide bonds) appear to be functionally important in the *in vivo* environment. These putative *in vivo* virulence factors include the trypsin-, thiol- and caseinolytic proteinases, and two

peptidases, glycylpropyldipeptidylaminopeptidase and glycylpropylpeptidase. If functional in the host, these enzymes could play a significant role in periodontal disease progression, including the dissemination of *P. gingivalis* and other bacterial species to deeper tissues of the host, resulting in tissue invasion and the destruction of host tissue and cells (128, 161, 215, 253, 328, 400, 420, 448, 449).

The classification of the proteinases has relied upon their catalytic functions. To date, four *P. gingivalis* proteinases are recognized: serine, aspartate, thiol and metalloproteinase. Of these, the collagenases, aminopeptidases, and the trypsin-like proteinases are critical to *P. gingivalis* pathogenesis. These latter proteinases have received the most interest during the past several years, and since they cleave polypeptides after arginine and lysine residues, they are classified as either arginine-(Arg-) or lysine-(Lys-) specific proteinases. The Arg- and Lys-proteinases are cysteine proteinases and have been given the common name, gingipains. The *P. gingivalis* collagenase has been classified as a proteinase with a hydrolytic predilection for collagen. The studies of Bourgeau et al. (32) suggest that these collagenases are cysteine proteinases.

Recent molecular biological approaches have indicated that, while there might be numerous cysteine proteolytic activities (generated from molecules with different molecular weights), there are at least three different genes which code for the proteolytic activity seen in *P. gingivalis*: these genes encode for two cysteine proteinases, arginine-gingipain (Arg-gingipain 1 and 2; RGP-1 and RGP-2) and a lysine-gingipain (Lys-gingipain 1; KGP) (324). Arg-gingipain is encoded by two genes, while Lys-gingipain is encoded by only one gene (KGP) (326). Sequence analysis has revealed that both the Arg-gingipain and the Lys-gingipain are unique proteinases, and do not share any similarity with any of the other known cysteine proteinase.

The *P. gingivalis* proteinases have been isolated from whole cells, cell extracts, cell sonicates, intracellular membrane-free material, intracellular membrane-bound material, outer membranes and their soluble products, outer membrane vesicles and spent growth supernatants. In essence, these proteinases have been isolated from all *P. gingivalis* cell fractions; however, the preponderance of the data indicates that these proteinases are concentrated at the surface of the cell. The distribution of enzymes in these various fractions has also been examined as a function of the growth cycle as well as a function of growth under different environmental conditions, including variations

Table 4. *Porphyromonas gingivalis* strain proteinases: localization and molecular weight

Strain	Location	Description	Molecular weight (kDa)	References
381	Cell envelope fraction	Dipeptidyl arylaminopeptidase	*	274
	Spent growth medium	Trypsin-like protease	*	459
	Spent growth medium	Glycylprolyl dipeptidylaminopeptidase	160	1
	Whole cells, soluble fraction	Caseinolytic protease-three isoenzymes	*	310
	Whole cells, spent growth medium	Trypsin-like protease	45-50	422
		Glycyl-arginyl peptidase-thiol protease	*	398
		Glycyl-prolyl peptidase-serine protease	*	
	Whole cells, spent growth medium	Thiol protease	*	345
	Spent growth medium	Trypsin-like protease	*	307
	Spent growth medium	Glycylprolyl aminopeptidase	75	90
	Cell sonicate fraction	Henagglutinin-cysteine protease	44	264
	Outer membrane fraction	Three caseinolytic protease 1	105-110, 72-80, 44	283
	Spent growth medium	Protease-Pase-S	43	153
	Spent growth medium	Trypsin-like protease	55	154
33277, 381, 1021		Arylaminopeptidase	*	25
	Outer membrane fraction	Glycylprolyl aminopeptidase	19.5, 29	397
	Whole cells	Collagenase	*	130
	Spent growth medium, outer membrane vesicle fraction, cell sonicate fraction	Proteases	110, 100, 90, 80, 65, 50, 30, 2	31
33277, E51	Cell sonicate fraction	Glutamate dehydrogenase (PgAgI)	51	181
	Spent growth medium	Argingipain-cystein proteinase	44	185
	Cell membrane fraction, spent growth medium	Cysteine proteinases-Arg-gingipain (RGPI), Lys-gingipain (KGP)	110, 95, 70-90, 50	328
	Spent growth medium	Trypsin-like protease	43	180
33277	Spent growth medium	Protease	300	102
	Cell sonicate fraction	Trypsin-like protease	35	394
	Cell sonicate fraction	Trypsin-like enzyme	35	424
	Intracellular membrane-free fraction, intracellular membrane-bound, extracellular fraction	Proteases	*	103
	Outer membrane vesicle fraction	Trypsin-like protease	80	124
	Spent growth medium	Glycosulfatase	37	377
	Cell membrane fraction	Lys-gingivain	70	355
	Whole cells, spent growth medium	Thiol-proteinase	140	67
	Spent growth medium, outer membrane fraction	Trypsin-like enzyme	64, 58, 11, 93, 70	380
	Whole cells, spent growth medium	Trypsin-like enzyme	58	385
W50	Whole cells, outer membrane vesicle fraction, spent growth medium	Trypsin-like protease	*	263
	Spent growth medium	Arg-x proteases-RI, RIA and RIB	*	334
	Spent growth medium	Arg-x proteases-RIIA and RIIB	*	335
	Spent growth medium	Chondroitinase-70, heparinase	200, 150	387
	Outer membrane vesicle fraction	Arg- and Lys-specific proteinase	300	27
	Cell sonicate fraction	Cysteine protease	*	358
W83	Spent growth medium, outer membrane vesicle fraction	Gingivain-cysteine protease	*	360
	Spent growth medium, outer membrane vesicle fraction		*	

Table 4. Continued

	Whole cells	Glycylprolyl peptidase	80	23
A7A1-28	Spent growth medium	Gingivain proteases	75, 70, 55, 80	24
9-14K-1	Spent growth medium	Gingipain-1 (RGP-1)	50	49
H66	Spent growth medium	Gingipain (hemagglutinin)	50, (44), 60	321
	Spent growth medium	Gingipain R1-gingipain R2-, gingipain K	95, 50, 105	170, 171

*Absence of reported data.

in growth media, growth environment (static, batch culture, continuous culture; see Environmental cues). Early studies reported the isolation of a large number of proteinases, which exhibited a wide range of molecular weights and substrate specificities (Table 4). At least 40 different proteinases have been described as being produced by *P. gingivalis* (324). For the most part, all of them function to cleave synthetic substrates containing either arginine and/or lysine. While there were significant differences in these proteins, the fact that they were isolated from a variety of strains grown under very different conditions and isolated by different procedures does not permit a comparison between these results. It is also apparent that none of these early studies followed the recommendations of the International Union of Biochemistry in the naming of these *P. gingivalis* "proteinases" (Table 4). Recent studies of the *P. gingivalis* proteinases have provided some consistency in both their identification and their potential function in the *in vivo* environment of a host. What has emerged from these latter studies is the biological importance of the two *P. gingivalis* cysteine proteinases, Arg- and Lys-gingipains (420).

Since the *P. gingivalis* proteinases are localized on the bacterial cell surface, mechanism(s) for their transport from the interior of the cell to the outer membrane must exist. Also, once associated with the outer membrane, the proteinases found within the vesicle are sometimes thought to be "excreted"; however, no data exists which indicates that *P. gingivalis* has a mechanism for the active secretion of these proteins (or outer membrane vesicles).

While it is apparent that more outer membrane vesicles appear in the external milieu as a function of the *in vitro* growth of *P. gingivalis*, in most of the *P. gingivalis* strains studied, the partially processed and active proteinase or hemagglutinin appears to remain associated with the cell surface in the form of a single polypeptide chain, or as a noncovalent complex between the two proteins. When these complexes are released into the extracellular environment, they are found in several forms associated with the outer membrane or outer membrane vesicles, or in a soluble multi-chain form. The hemagglutinin domain of gingipain R1 is repeated four times in the *hagA* gene (139 and see below). An interesting feature of the *hagA* gene is the occurrence within its structure of several large contiguous direct repeats. The most significant of these repeats is a 50 amino acid structure which is repeated three times in *rgp-1* and *kgp*, two times in the porphypain, and five times in *hagA* gene (139). It has been postulated

that these repeats are involved in *P. gingivalis* hemagglutinin activity; however, the exact location of their active sites is still to be determined.

Movement of the *P. gingivalis* cysteine proteinases formed in the bacterial cytoplasm to the outer membrane requires the intervention of a secretory pathway. The secretory system apparently involved in this transfer of the gingipain protein from the cytoplasmic region to the outer membrane is very similar to the IgA1 secretory pathway found in *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Haemophilus influenzae*, the serine protease in *Serratia marcescens*, the vacuolating cytotoxin of *Helicobacter pylori*, the SepA secreted protein from *Shigella flexneri* and the 110-kDa secreted EspC protein of the enteropathogenic *E. coli*. All of these proteins are secreted via an outer membrane-anchored intermediate (94, 184, 206). This secretion mechanism is typical for proteins which mediate their own secretion, and are secreted by a type IV secretion mechanism (94). Proteins which are secreted by this type IV secretion mechanism are able to direct their own movement across the outer membrane and function as auto-transporters by forming pores in the membrane through which they pass. In several of the *P. gingivalis* strains, gingipain R1 remains attached to the outer membrane, while in other strains it is shed into the environment as a result of bacterially associated proteolysis (49, 321, 324, 334). In *P. gingivalis* strain W12, for example, a single chain, membrane-associated, high-molecular-weight mass form (120 and 150 kDa) of arg and lys-specific cysteine proteinases is typically isolated (54). Employing Western blot analysis (324) using anti-HRGP antiserum and confirmed by specific labeling of the active cysteine residue, determined that the membrane-associated enzymes contain an extra 15-kDa polypeptide that is thought to be involved in outer membrane anchoring.

Identical to other proteins transported via this type IV secretion mechanism, the gingipains are derived from precursors which are substantially larger than the secreted products. Structurally, the gingipain R1 polyprotein is similar to the *Neisseria* IgA1 protease precursor, including an *N*-terminal signal polypeptide, followed by a domain corresponding to the mature secreted proteinase, and a *C*-terminal extension. This *C*-terminal extension of the IgA1 protease harbors secretory functions for outer membrane transport.

Trypsin-like proteinase (the gingipains) from *P. gingivalis*. Among the early investigations into the ex-

pression of *P. gingivalis* proteinases, Sawyer et al. (346) first reported that the black-pigmented *Bacteroides* strains (originally *Bacteroides gingivalis*), produced a lytic proteinase capable of hydrolyzing proteins. Two decades later, Slots (378) and others (80, 197, 222, 223, 413) using a semiquantitative enzyme identification assay (API ZYM) reported that, in fact, *B. gingivalis* (*P. gingivalis*) possessed very strong trypsin-like proteolytic activity against the synthetic substrate, benzoyl-D,L-arginine- β -naphthyl-amine (BANA). Hydrolysis of this substrate resulted in the separation of the original *B. gingivalis* and other black-pigmented *Bacteroides* species from the now recognized *P. gingivalis*.

Yoshimura et al. (456) localized a partially purified trypsin activity (*B.* *P. gingivalis*) in "membrane-associated material". These investigators also demonstrated that this *P. gingivalis* trypsin proteinase was significantly upregulated by -SH reagents and also inhibited by -SH inhibitors. A protein of similar function was also isolated and apparently purified by Fujimura & Nakamura (102), Tsutsui et al. (422) and Ono et al. (307), from the spent growth medium and from a "membrane-free fraction". These purified proteinases had molecular weights of 65, 300 and 45 to 50 kDa. A trypsin proteinase with similar molecular weights (64 and 58 kDa) was also isolated from the spent growth medium from *P. gingivalis* strain W50 and from cell sonicates (35 kDa) by Smalley & Birss (380) and Sorsa et al. (394), respectively. Separate protein(s) with trypsin hydrolytic activity was also described by Endo et al. (90) from *P. gingivalis* culture supernatants. This material was very active against reduced lysozyme, with the cleavage site being identified as the carboxyl region of arginine. Fujimura & Nakamura (103) fractionated *P. gingivalis* into various cell fractions and isolated several trypsin enzyme activities. While the various cell fractions contained variable proteinase activities, their enzymatic properties were very similar, and were, more than likely "multiple-forms" of the same enzyme.

Marsh et al. (251) were the first to systematically identify the extent of the trypsin activity from *P. gingivalis*. They compared this activity from *P. gingivalis* strain W50 with the naturally occurring weakly pigmented, colonial variant, W50/BE1. The parent strain had significantly greater trypsin activity (three times) than the variant.

Several studies have attempted to quantitate the level of the *P. gingivalis* trypsin enzyme, as well as the periplasmic-associated enzymes alkaline phosphatase and *N*-acetyl- β -glucosaminidase from chemostat maintained cultures of *P. gingivalis* (263,

381). *P. gingivalis* W50 and W50/BE1, were grown under chemostat maintained conditions. Outer membrane vesicles, and growth supernatant were isolated and assayed for the distribution of enzyme activity. Significant activity of the two enzymes was found to be associated with the outer membrane vesicles. Interestingly, the levels of these enzymes was higher in whole cells during exponential growth than that found in the vesicles as well as in the soluble spent culture supernatant. Quantitatively, almost 90% of the trypsin activity was associated with the parent strain, while only 10 to 30% of a similar activity was associated with the nonpigmented variant, W50/BE1. The protein content in the vesicle fraction of the variant was significantly higher than that produced by the parent strain (386).

The cysteine proteinases are thiol proteinases and comprise the group of endopeptidases whose members rely for their catalytic activity on the presence of the thiol group of a cysteine residue in the enzyme molecule. The other three main classes of endopeptidases are the serine proteinases, aspartate proteinases and the metalloproteinases. The cysteine proteinases have been isolated from a large number of biological sources, including plants, animals and bacteria.

Most of the cysteine proteinases are small molecules, with molecular weights in the range of 20,000 to 35,000. Several proteases with molecular weights larger than 35 kDa have been reported and include the calpains (110,000) and clostripain (55,000). The isoelectric points of the various cysteine proteinases cover a wide range from 3 to 11.7. For the most part, these proteinases catalyze the hydrolysis of a variety of polypeptide substrates (acyl compounds with different specificities and involved in a wide range of physiological processes). To date, all of the cysteine proteinases studied exhibit endopeptidase activity towards proteins and polypeptide substrates; several of these display dipeptidylcarboxypeptidase and aminopeptidase activity.

The secreted cysteine proteinase has also been shown to lyse red blood cells (359–361). These investigators were successful in purifying this hemolytic protein and called it gingivain. Gingivain was in fact a hemolysin, being capable of the rapid lysis of red blood cells. The purified protein was not neutralized by antiserum to the cloned *P. gingivalis* hemagglutinin, indicating that this activity was independent of the hemagglutinin. The anti-hemagglutinin antibody was effective in inhibiting hemagglutination activity, and the two proteins were therefore considered to be separate proteins with at least two independent

activities. Scott et al. (355) have also isolated and characterized a Lys-gingivain from *P. gingivalis* as a thiol proteinase. It functions as a lysyl-amidase with a molecular weight of 70 kDa. It is a potent kininogenase and fibrinase and is capable of increasing the coagulating activity of the high-molecular-weight kininogen, releasing bradykinin and low-molecular-weight kinogen, resulting in the destruction of the light chain (that is, coagulant portion) of kininogen (see below). Subsequently, another arginine-specific cysteine proteinase with a molecular weight of 44 kDa was isolated and given the trivial name argingivain. Argingivain is a thiol-dependent proteinase with characteristics very similar to both metallo and serine endopeptidases. Interestingly, the normal host proteases appear to have little, if any effect on this thiol-protease, suggesting its ability to evade at least one host defense (185).

Bedi (24) purified four gingivain thiol-activated arginine- and lysine-specific proteases from *P. gingivalis* culture supernatant and named these arg-gingivain-75, arg-gingivain-70, arg-gingivain-55, and lys-gingivain-80 kDa based on their molecular masses and specificity for either arginine or lysine in the P1 position. A major cell-associated Arg- and Lys-specific cysteine proteinases with a molecular weight of 300 kDa complex consisting of seven fractions (48, 45, 44, 39, 27 and 15 kDa) by SDS-PAGE was recently described and a 44-kDa fraction containing two different proteins characterized by N'-terminal sequence analysis (27). The 45-kDa protein of the complex was purified and shown to be an Arg-specific, thiol-activated, calcium-stabilized cysteine proteinase. Similarly, the 48-kDa protein was also purified and shown to be a Lys-specific cysteine proteinase that was not inhibited by EDTA. The two 44-kDa and 39-, 27-, 17- and 15-kDa proteins of the complex exhibited amino acid sequence homology and have been proposed to be hemagglutinins/adhesins. The 45-kDa Arg-specific adhesins are processed from the single polypeptide encoded by the gene *prtR*. Similarly, the 48-kDa Lys-specific proteinase, the 39-kDa and 15-kDa adhesins as well as the other 44-kDa adhesin of the 300-kDa complex are encoded by a single gene, *prtK*. Three forms of extracellular arginine-specific proteases (Arg-x proteases) RI, RIA and RIB, were isolated from *P. gingivalis* and were all derived from *prpRI* gene expression. Both RIA and RIB are monomeric species, RI is a heterodimeric enzyme composed of an α -component (54 kDa), which bears the protease active site, and a β -component (58 kDa) (334). Based upon their physical properties and kinetic behavior, RIA appears to

be analogous to gingipain from *P. gingivalis* HG66. Inactivation of the *prpR1* gene from an isogenic mutant enabled the detection in the culture supernatant of two additional related Arg-x proteases (RIIA and RIIB), identical to RIA and RIB. *prR2* was cloned, sequenced, and found to occur at a second genomic locus. These data indicate that RI, RIA and RIB are produced by *prpR1* expression and a maturation pathway. Similarly, RIIA and RIIB, the products of *prR2* are exported into the culture supernatant in the absence of *prpR1* expression (335).

DeCarlo & Harber (67) isolated a thiol-dependent proteinase (140 kDa) from *P. gingivalis* cells and spent growth supernatant and found it to consist of noncovalently associated peptides. The slight variations in the association pattern of these peptides could have resulted in different proteinases (18–20, 32–35, 42, 48–50, 52, 80 and 106 kDa) with different affinities and activities. Western blot analysis showed that many of these proteinases with varying molecular weight are related and originated from a single immunoreactive 140 kDa proteinase. This proteinase activated host-degradative enzymes such as matrix metalloproteinases (MMPs) MMP-1, MMP-3, and MMP-9 and was observed to catalyze the superactivation of MMP-1 by MMP-3.

Studies on hydrolytic enzyme levels and their distribution in *P. gingivalis* batch culture showed that whole cells and outer membrane vesicles exhibited consistently high trypsin-like protease, alkaline phosphatase, acid phosphatase, *N*-acetyl- β -glucosaminidase and collagenolytic activities (195). These investigators found that both trypsin, alkaline phosphatase and *N*-acetyl- β -glucosaminidase were significantly higher in whole cells and outer membrane vesicles than in any of the other soluble or insoluble fractions, including spent culture supernatant.

Ciborowski et al. (54) and Lantz et al. (219–221) purified two outer membrane-associated cysteine proteinases from *P. gingivalis* strain W12, the molecular weights of which were 150 and 120 kDa. These proteins, which Lantz et al. (220, 221) named porphypain-2, possessed reducing agent enhanced fibrinolytic activity and were also inhibited by -SH-blocking agents. These biochemical characteristics are similar to the other *P. gingivalis* cysteine proteinases so far isolated and studied. The ability of porphypain-2 to cleave fibrinogen *in vivo* could have a significant effect on the progression of a periodontal infection. Employing this enzyme, *P. gingivalis* could, for example, degrade extracellular matrix components, resulting in an enhanced invasion of the deeper periodontal tissues of a host. Activation of

this cysteine protease could also inhibit the repair of host tissue proteins, providing small peptides for the growth and survival of this bacterium (and others) in the confines of the periodontal environment. In a recent study, Moritz et al. (266) examined the ability of purified porphypain-2 to protect the nonhuman primate *Macaca fascicularis* from attachment and alveolar bone loss in the ligature-induced periodontitis model of Kornman et al. (209). Porphypain-2-immunized animals had a 200-fold increase in IgG and a small, but significant difference in protection from alveolar bone density loss compared with control, placebo-immunized animals. Thus, porphypain-2 could be an important immunogen, and further studies will be required to extend these initial observations.

In several recent studies, Genco et al. (113) have employed the mouse chamber model to study the effect of several proteinase inhibitors on *in vivo P. gingivalis* activity. Preincubation of *P. gingivalis* with the proteinase inhibitors leupeptin, Z-FKcK, or Z-Fack prior to *P. gingivalis* mouse challenge resulted in the inhibition of abdominal lesion formation. Identical incubation of *P. gingivalis* with these inhibitors also resulted in growth of the bacterium in the chambers, indicating that inactivation of the cells proteinases, including the gingipains (R and K) prior to mouse challenge, results in a decrease in *in vivo P. gingivalis* virulence. While it remains to be determined how these proteinases function in the *in vivo* environment to influence *P. gingivalis* virulence, it is possible that they have several functions, including influencing adherence to host tissues and cells, degrading host protective proteins and, most importantly, interfering with host defenses (112).

Genco et al. (113) have recently provided some very interesting and provocative results that address the role(s) of a *P. gingivalis* synthesized protein (gingipain) in *in vivo* virulence. They hypothesized that antibody to the gingipains function to protect the host from *P. gingivalis* infection in their mouse chamber model. Mice were immunized with either *P. gingivalis* whole cells or purified gingipain R1 or R2, followed by bacterial challenge. Immunization protected the animals against both *P. gingivalis* colonization and invasion. Both immunogens (whole cells and R1, R2) generated a significant IgG response that was targeted against epitopes within the adhesin and hemagglutinin domain of gingipain R (113). The hemagglutinin domain of the gingipain appears to function as a major *P. gingivalis* antigen. Since repeated amino acid sequences within the hemagglutinin domain are also present in the *P. gin-*

gingivalis *hagA* gene product and in the *tla* gene product, the strong antibody response observed to the hemagglutinin domain may be due to the immunogenicity of these amino acid repeat sequences found within the hemagglutinin domain (see below). Genco et al. (112) also observed that while there was strong immune reactivity to the hemagglutinin domain of gingipain R1 and K, very little reactivity was observed to these catalytic domains using anti-gingipain R1 raised in rabbits or chickens, suggesting that the amino acid sequences within the hemagglutinin domain of the gingipains are very immunogenic.

Chen et al. (49) isolated a cysteine proteinase from the outer membrane of *P. gingivalis* that had a molecular weight of 50 kDa and required both cysteine and Ca^{++} for activity and stabilization. They termed this proteinase as gingipain-1, and amino acid and nucleotide sequence analysis revealed that, while some similarities to the other *P. gingivalis* proteinases existed, the proteins were different enough to provide them with several unusual features, including the stimulation of amidolytic activity by glycine-containing dipeptides, a very narrow proteolytic specificity to peptide bonds containing arginine and lysine residues and a profound resistance to inhibition by host proteinase inhibitors. Therefore, gingipain-1 and gingipain-2 are different proteins, and their differences have been confirmed by Pike et al. (321) and Potempa et al. (326), who isolated and characterized two separate cysteine proteinases from *P. gingivalis*, each containing specific proteolytic activities: a 50-kDa arginine-specific enzyme and a 60-kDa lysine-specific one. Both the 60-kDa lysine-specific enzyme (Lys-gingipain) and the 50-kDa arginine-specific enzyme (Arg-gingipain) had lower-molecular-weight peptides (40 kDa) associated with them that appeared to function in hemagglutination through a hemagglutination-binding protein. Western blot analysis using anti-arginine-X and lysine-X-specific proteinases has revealed that RGP and KGP exist in different cell fractions as multiple-molecular-weight species (110, 95, 70 to 90, and 50 kDa) in several of the *P. gingivalis* strains and that these protein species more than likely are the result of the proteolytic processing of the initial polypeptide transcripts from *rgp1*, *rgp2* and *kgp* genes which encode two arginine-specific and one lysine-specific gingipain (314, 324). The three forms of the gingipains [gingipain R1 (95 kDa), gingipain R2 (50 kDa), and gingipain K (105 kDa)] were originally characterized in terms of their interaction with multiple synthetic peptidyl chloromethane inhibitors and peptidyl (acyloxy)methanes. The chlorome-

thane inhibitors were strongly inhibitory to all of the gingipains, with the extent of inhibition dependent on the peptidyl components of the inhibitor. The (acyloxy)methane inhibitor produced a very specific and rapid inhibition of gingipain K and R. Gingipain 1 or 2 were always present in *P. gingivalis* in at least a three-fold excess compared to gingipain K, with the gingipains accounting for at least 85% of the total *P. gingivalis*-associated proteolytic activity (327).

The two large cysteine proteinases, porphypain-1, porphypain-2, were purified to homogeneity with molecular masses of 150 kDa and 120 kDa, respectively. Both had Arg-X and Lys-X specificity but at different active sites on the basis of inhibitor studies (54). The gene encoding these unusual proteinases was cloned and sequenced (21).

An arginine-specific thiol-dependent cysteine proteinase (44 kDa) argingipain was also isolated from *P. gingivalis* and found to extensively degrade collagens (type I and IV) (185), whereas gingipain-1, a 50-kDa cysteine protease, has no collagenolytic activity (49). In contrast, a purified trypsin-like proteinase with a molecular weight of 55 kDa was isolated from the spent growth medium and found to hydrolyze type I, III, IV, and V collagen (connective tissue components of human periodontal tissue) and C3 complement components, fibrinogen, fibronectin, α_1 -antitrypsin, α_2 -macroglobulin, apotransferrin and human serum albumin (components of host defense mechanism) (25).

It appears now that the trypsin enzyme originally isolated and partially characterized by Sawyer (346), from *P. gingivalis* is the cysteine proteinase we now refer to as gingipain. The available data also indicate that *P. gingivalis* produces arginine- and lysine-specific cysteine proteinases and that these are synthesized as polyproteins containing a prepropeptide, a catalytic domain and a hemagglutinin domain. These precursor proteins are post-translationally processed, which results in the production of different forms of the proteinases by *P. gingivalis*.

Functions of *P. gingivalis* proteinases. While the exact mechanism(s) by which Arg- and Lys-gingipains (or any of the other proteinases from the oral bacteria) function, especially *in vivo*, remains to be determined, a variety of observations indicate that these proteinases from *P. gingivalis* are involved in a variety of functions, including adherence to host cells, to other bacteria in the host environment, to supporting *in vivo* growth of *P. gingivalis* as well as to inhibiting selected host defense mechanisms. While some of the data are still to be confirmed and ex-

tended, several investigators have postulated that *P. gingivalis* proteinases might also be involved in direct tissue destruction. The gingipains have been shown to be potent vascular permeability upregulators, being capable of inducing vascular permeability in human plasma and cleaving bradykinin directly from the high-molecular-weight kininogen (169, 171). This ability to significantly increase gingival vascular permeability in periodontitis sites results in increased gingival fluid flow. Since the gingipains are chemotactic for polymorphonuclear leukocytes, there is an increased concentration of these host cells at sites of potential tissue and bone destruction. Associated with polymorphonuclear leukocyte function are the observations that *in vitro* *P. gingivalis* is capable of degrading complement C3, and interfering with phagocytic events elicited by polymorphonuclear leukocytes. One of these important host-defense mechanisms is the ability of the polymorphonuclear leukocytes to generate active oxygen species, which are bactericidal in the host. The observations to date (185) indicate that the *P. gingivalis* Arg-gingipain is the protein responsible for this inhibition. In fact, *P. gingivalis* is the only prokaryote so far reported to be capable of inhibiting this polymorphonuclear leukocyte function. While the *in vivo* data still need to be confirmed, it is clear that, with the accumulation of these neutrophils in the confines of the developing periodontal pocket, there is the potential for these host cells to activate and secrete numerous host destructive enzymes such as the elastases, cathepsins, gelatinases, and collagenases. All of these may participate (along with the *P. gingivalis* proteinases) in the destruction of periodontal tissues.

Childs & Gibbons (50), Naito & Gibbons (273) and Hesketh (152) have reported that several of the *P. gingivalis* proteinases might function in the host to degrade cell membranes to uncover hidden binding sites, or cryptitopes. Several of these enzymes are also thought to be involved in a direct binding of the bacterium to host tissue via an active site on the enzyme. In the reports by Gibbons et al. (120), they demonstrated that treatment of epithelial cells as well as fibronectin-collagen complexes with trypsin resulted in a significant increase in the binding of *P. gingivalis* to these cells.

In *in vitro* experiments, a large number of proteins have been found to be degraded by the proteolytic enzymes from *P. gingivalis*. These proteins include both human and animal collagen (340, 415), casein (310), gelatin (252), fibrin (220, 221, 284), immunoglobulins (129, 185, 198, 402), complement factors

(347, 401–403), inactivation of leukocyte C5a receptor on neutrophils (179), proteinase inhibitors (42, 123, 185), iron transport proteins (43), plasma proteins (61, 95) as well as several synthetic substrates.

Previous studies of the mechanism of proteinase action on the insulin β -chain revealed that the *P. gingivalis* proteinase was capable of cleaving peptide bonds at both the C-terminus of arginine and lysine, while trypsin cleaved at the N-terminal of arginine, and the C-terminus of glutamine, glutamic acid and alanine (89, 157). The *P. gingivalis* proteinases were also capable of the rapid depolymerization of human plasma fibronectin. Similar activity against fibronectin was also observed with *P. gingivalis* outer membrane vesicles. This latter activity may be one of the most important virulence factors in the dissemination of *P. gingivalis* throughout the host. They could provide a significant invasive capacity, since fibronectin is a major participant in the maintenance and remodeling of the periodontal ligament (385).

Some of the first studies of the effect of *P. gingivalis* trypsin enzymes on human gingival fibroblasts employed a partially purified trypsin enzyme with a molecular weight of approximately 35 kDa. This impure enzyme resulted in the rapid degradation of an intracellular fibrinogen matrix and resulted in the secretion of increased amounts of collagenase and plasminogen activator (424). Since this mixed enzyme displayed greater activity than purified trypsin alone, there were more than likely several enzymes that co-purified with the trypsin molecule. However, in spite of the fact that the Uitto et al. (424) enzyme was more than likely mixed, the data does support the role of proteolytic degradation *in vivo* as an important mechanism for the destruction of supporting tissue and bone.

P. gingivalis also has the capacity to produce proteinases that are capable of protecting it from several host defense mechanisms. For example, *P. gingivalis* produces several immunoglobulin proteinases. IgA1, IgA2, and IgG proteinases capable of cleaving IgA1 at the hinge region of the immunoglobulin, producing intact Fc $_{\alpha}$ and Fab $_{\alpha}$ fragments (99, 198, 199). These microbial endopeptidases are also able to degrade human colostrum (human colostrum IgA) and secretory IgA (345). Functionally, while it is still uncertain as to the role of these IgA proteinases in *P. gingivalis*, it has been shown that several *P. gingivalis* strains were able to degrade nonspecific human IgG and IgA into smaller fragments which strongly stimulated bacterial growth *in vitro*. Not only were these immunoglobulin fragments significantly stimulatory to *P. gingivalis* growth, but they also pro-

tected the bacteria from reaction with anti-*P. gingivalis* antibody. In fact, Grenier et al. (129) demonstrated quite convincingly that, while anti-*P. gingivalis* antibody bound to the bacterial surface, the cells themselves were able to hydrolyze this bound antibody to smaller inactive fragments. Therefore, *P. gingivalis* might be capable of reducing the concentration of active IgG in gingival fluid in the localized environment of the gingival sulcus, resulting in the localized growth of this important member of the periodontopathic microbiota. None of the *P. gingivalis* proteinases was susceptible to the host proteinase inhibitors α_2 -macroglobulin and α_1 -proteinase inhibitor, and therefore, these *P. gingivalis* proteinases might be central to the destruction of host tissue (99).

Metabolically, the ability of *P. gingivalis* to produce a variety of proteinases could be essential for its survival in a host. Since it is an asaccharolytic bacterium unable to metabolize carbohydrates, it must rely for its carbon and energy sources from proteins. Thus, the ability of the trypsin proteinase to degrade large proteins into small peptides provides a potentially important mechanism for the growth and multiplication in the host (129, 362). Importantly, since *P. gingivalis* also requires iron-containing molecules (hemin) for growth, its ability to cleave large proteins (transferrin and hemoglobin), might provide a mechanism by which *P. gingivalis* secures its essential iron (43). Outer membrane vesicles were also capable of completely inactivating the bactericidal activity of human serum. A similar inhibition was also observed with *P. gingivalis* lipopolysaccharide, suggesting that the lipopolysaccharide and proteolytic enzymes were involved in this phenomenon (125).

P. gingivalis also contains several caseinolytic proteinases which have been isolated from the culture supernatant. These proteinases were capable of degrading such mammalian proteins as type I and IV collagen, human IgG, fibronectin (385, 424), and complement C3, C4, C5, and C5a (73, 124, 157, 179, 347, 401–403, 441, 448), as well as inhibiting the bactericidal activity of polymorphonuclear leukocytes (185, 448, 453). Gingipain R (protease I) was also found to cause a true platelet activation at concentrations comparable to thrombin (61).

P. gingivalis is also capable of hydrolyzing or degrading a number of host plasma proteinase inhibitors (42, 99, 123, 151, 153, 282), such as α_1 -antitrypsin and α_2 -macroglobulin, C₁-inhibitor, α_2 -antiplasmin, antithrombin, α_1 -proteinase inhibitor (99). *In vivo*, these plasma proteinase inhibitors could func-

tion to enhance host tissue inflammation via the activation of host cytokine synthesis.

Binding studies suggest that gingipain R and gingipain K, acting as proteinase-adhesin complexes, progressively attach to, degrade and detach from target proteins (322). The arginine-specific cysteine proteases (gingipain Rs) with molecular weights of 95 and 50 kDa activate coagulation factor X in a dose- and time-dependent manner, suggesting that factor X activation by gingipain Rs could occur in plasma (172). Both 50- and 95 kDa gingipain R were efficiently inhibited by human plasma α_2 -macroglobulin (abundant in vascular fluids), whereas the catalytic activity of gingipain K could not be eliminated. All three enzymes were, however, inhibited by rat plasma macroglobulin, which was different from human macroglobulin in amino acid sequences (132). Molecular studies of the close structural relationship between cysteine protease and hemagglutinin activities of *P. gingivalis* using isogenic mutant G-102, deficient in Arg-gingipain displayed reduced protease and significant hemagglutination activity indicated that both activities can be expressed from a single gene (452). Further studies also showed that this mutant is deficient in attachment properties to human oral epithelial cells, extracellular matrix proteins and type I collagen, defective in interacting with early colonizing gram-positive bacteria (*S. gordonii* and *A. viscosus*), decreased autoaggregation, altered in normal fimbria expression, and has reduced expression of the major 43-kDa fimbriin subunit (214, 416). Recently, a mutant defective in the *rgpA* gene, MT10, was constructed in *P. gingivalis* strain 381. This mutant displayed reduced Arg-specific cysteine proteinase activity, self-aggregation, hemagglutination and binding to immobilized type I collagen compared with the levels in the wild-type parent. However, this mutant displayed increased binding to epithelial cells and did not express any detectable levels of the FimA protein (417).

Proteinases and periodontal disease. While a large number of studies have examined the types, numbers and distribution of microorganisms found in health and in the progression to periodontitis, the actual role of these resident microbiota in this progression to disease is still unclear. Haffajee & Socransky (134) have provided the most seminal data indicating that bacterial consortia provide the necessary "biological balance" for the maintenance of health. Shifts in this balance to an unfavorable one results in the growth and emergence of another complex consortium characteristic of disease.

Several studies have attempted to correlate the human antibody responses (serum levels of IgG, IgM and IgA) to selected periodontal pathogens and the progression of periodontitis. These studies have proposed to implicate specific bacterial species, as well as specific cell components (lipopolysaccharide, outer membrane proteins, fimbriae and specific proteinases) in this progression (85, 87). These investigators found a statistically significant correlation between the antibody levels of selected oral species and disease severity. Ando (15), also observed a significant positive correlation between the antibody levels of dipeptidylaminopeptidase (IV) in gingival crevicular fluid and the clinical severity of periodontitis. Similar observations were made by Ismaiel et al. (174), who studied the relationship between the antibody levels against the *P. gingivalis* trypsin-like enzyme in patients diagnosed with adult periodontitis and rapidly progressive periodontitis compared with healthy control individuals. The diseased patients had significantly higher levels of specific IgG, IgM and IgA antibodies against purified trypsin-like enzyme from *P. gingivalis* spent culture supernatants compared with control healthy subjects. It is more than likely, then, that the host is processing the *P. gingivalis* trypsin protein as an immunogen. Similarly, Suido et al. (399) found a significant positive correlation between the levels of gly-propeptidase activity in crevicular fluid and the number of *P. gingivalis* cultured from the periodontal environment. Therefore, the evidence is convincing that there is a positive relationship between the antibody levels of selected bacterial enzymes as a function of periodontal disease progression.

Role of *P. gingivalis* proteinases: *in vivo* virulence. The question still remains as to the role of the *P. gingivalis* virulence factors (cell components and enzymes), in the *in vivo* destructive activity of periodontal inflammation, bone and tissue destruction. However, in order to address the *in vivo* question of selected interactions between oral bacterial and host cells, several animal models, including rat and murine models and the murine lesion model (253), have provided important information (see section on animal models).

Subcutaneous injection of mice with *P. gingivalis* whole cells produces a spreading phlegmonous lesion that results in the destruction of soft tissue (86, 194, 253). The extent of lesion formation (size) is a function of the individual *P. gingivalis* strains. Strain ATCC 53977, for example, produced the largest of the lesions, while strain 381 was the least

"virulent" and did not produce secondary lesions (86). Cellular components, such as the lipopolysaccharide, soluble cytoplasmic material and even fimbriae appeared to be ineffective in either enhancing or protecting against lesion formation. McKee et al. (258) showed that the nonpigmented variant W50/BE1 was less virulent in this animal model than its parent strain.

Recent reports have attempted to address the role of several of the *P. gingivalis* enzymes as potential virulence factors in lesion formation in the murine model. Chemical, isogenic mutants and spontaneous nonpigmented, poorly pigmented *P. gingivalis* mutants deficient in trypsin formation as well as hemagglutination and collagenase mutants were used in these studies. Fletcher et al. (98) were one of the first to report that a *P. gingivalis* W83 mutant in which the *prtH* gene (an RGP-1 analog) was inactivated had reduced proteolytic activity, as well as being significantly less virulent in the murine model. This mutant also produced reduced degradation of complement-derived opsonins. When opsonization did occur, the mutant was engulfed by human polymorphonuclear leukocytes in significantly greater numbers than its parent (348).

Kesavalu et al. (196, 197) have provided evidence that the *P. gingivalis* trypsin proteinase is a major contributor to both lesion formation and death in the murine lesion model. These investigators compared wild-type *P. gingivalis* to trypsin-negative (NG4B19) or trypsin-reduced (W50/BE1) mutants and wild-type strains in the murine lesion model. The trypsin enzyme negative mutant did not produce any lesions, while the trypsin-deficient mutant produced only a localized soft tissue lesion. Therefore, the trypsin enzyme appears to be closely correlated with lesion formation (soft tissue destruction), and even animal death.

While the literature is clear that the progression from gingival health to periodontal disease is accompanied by both an increase in the numbers of gram-negative bacteria and a significant increase in proteolytic activity in the periodontal niche, there is unfortunately still a paucity of information on the role of the host in periodontal destruction. Since several of the bacterial enzymes are indistinguishable from host enzymes, discriminating between the activation of the numerous host proteinases and those contributed by the resident periodontal pathogens is unclear. Therefore, it is difficult to unequivocally invoke bacterial (*P. gingivalis*) proteinases as essential bacterial host degradative enzymes since very few published reports have provided convincing

data correlating the increase in *P. gingivalis* with increasing inflammation and the destructive events of periodontal disease. However, Ismaiel et al. (174) have demonstrated that *P. gingivalis* proteinases might be functional in human hosts. They observed that the serum IgG activity in periodontitis patients was significantly increased in the serum IgG-proteinase responses as a function of disease activity. Thus, they postulate that the *P. gingivalis* proteinases are important in the progression of disease.

Finally, in a very provocative report, Travis et al. (421) postulate that the numbers of *P. gingivalis* in a developing periodontal site might control both tissue and bone destruction and the growth of the other periodontal pathogens within the confines of the periodontal sites. With the increased growth of *P. gingivalis* (as a function of disease progression), there is a concomitant increase in the levels and secretion of the cysteine proteinases. The increase in the levels of these enzymes is such that the host's ability to neutralize them by plasma proteinase inhibitors is overwhelmed and non-functional. The functional bacterial proteinases are therefore capable of activating both the complement cascade and the necessary chemotactic events necessary for the migration of polymorphonuclear leukocytes and other inflammatory cells into the periodontal site. The ability of *P. gingivalis* to elicit polymorphonuclear leukocyte-inhibiting enzymes then results in nonfunctional phagocytic cells and the survival and growth of the now pathogenic microbiota. This continued growth and multiplication of these bacteria (especially *P. gingivalis*) results in the significant influx of inflammatory cells and their release of host destructive enzymes, with resultant periodontal damage and tissue destruction.

Aminopeptidases. The aminopeptidases have been found in several members of the oral microbiota, many of which exhibit some arylaminopeptidase activity. However, *P. gingivalis* to date is the only member of this periodontopathic microbiota that exhibits strong dipeptidyl arylaminopeptidase activity (274, 397). Abiko et al. (1) purified dipeptidylaminopeptidase from the spent growth supernatant of *P. gingivalis* and exposed it to type I collagen, cleaving a glycylpropyl dipeptide from the collagen protein. While the original studies of Nakamura et al. (274), defined a "pure enzyme", further studies of this aminopeptidase revealed that it contained at least two additional aminopeptidases, *N*-CBz-glycyl-arginyl peptidase (*N*-CBz-Gly-Gly-Arg), which was both cell associated and extracellular, and an extracellular

peptidase, glycyl-prolyl peptidase (Gly-Pro peptidase). Both aminopeptidases were thiol and serine activated proteinases, respectively (398).

Grenier & McBride (130) have purified a Gly-Pro aminopeptidase from the *P. gingivalis* ATCC 33277 outer membrane and have purified the protein to homogeneity with a molecular weight of 29 kDa. Interestingly, Miyauchi et al. (264) and Barua et al. (23) also purified at least two similar aminopeptidases from *P. gingivalis* cell extracts; however, these investigators report that these purified proteins had molecular weights of 75 and 80 kDa. Clearly, considerably more studies will be required to understand the differences in molecular weight and whether there are in fact at least three separate aminopeptidases present in *P. gingivalis*.

Grenier & McBride (131) were successful in localizing their aminopeptidase activity to the surface of *P. gingivalis*. Immunoelectron microscopy localized the enzyme in the periplasmic space. The aminopeptidase they isolated in this study appeared to be a much larger protein than the 29-kDa aminopeptidase they originally isolated. This latter protein was reported by Kay et al. (191) to have a molecular weight of at least 160 kDa, and the activity was also found associated with outer membrane vesicles, and in an extracellular protein fraction, the latter more than likely the result of membrane or vesicle lysis.

***P. gingivalis* caseinases.** Caseinases hydrolyze the protein casein and exist in *P. gingivalis* as at least three isoenzymes (310). They are active against salivary and egg-white lysozyme and insulin chain B. These three caseinolytic proteinases, called Pase-A, -B and -C, display different cleavage patterns. Pase-B and Pase-C were identified as thiol-proteinases and displayed a cleavage pattern similar to that of trypsin (that is, cleavage on the carboxyl side of arginine), and Pase-A was identified as a serine proteinase, which had as its preferred cleavage site the carboxyl side of lysine (153, 157). Immunological characterization suggested that Pase-C is located on the cell surface and monoclonal antibodies against the protein inhibited the hemagglutinating activity, hydrolyzed BApNA and strongly degraded native type I collagen (155, 156). These investigators also isolated and purified a 43-kDa thiol-dependent extracellular soluble protease (Pase-S) possessing BApNA-hydrolyzing activity (154). This enzyme resembled Pase-B in its hydrolytic specificity, cleaved only arginine residues of peptides, degraded type IV and denatured type I collagen. The enzyme was less active than Pase-C and might be an isozyme of Pase-B (155).

Collagenase. Collagen has been found to comprise a significant percentage of gingival connective tissues. The major portion of the gingival connective tissue is the highly organized collagen fibers, which have as their primary function the maintenance of tissue integrity and function (423). The clinical hallmark of periodontal disease is the destruction of the periodontal connective tissue during inflammation. Since mammalian collagen (type I) is resistant to destruction by the majority of the mammalian and bacterially synthesized proteolytic enzymes, the periodontal tissue destruction observed during the temporal events of periodontal disease must be mediated by specific proteolytic enzymes, especially the collagenases. While both mammals (including humans) and members of the periodontopathic microbiota (*P. gingivalis*) produce collagenase, either of these enzymes might contribute to the destruction of the supporting tissues of the teeth; however, several studies (423) have demonstrated that the collagenolytic activity involved in periodontal tissue destruction predominantly involves the host-derived enzyme. Data support the participation of bacterially derived collagenase (*P. gingivalis*) in connective tissue destruction; however, these data are still equivocal. Collagenase is perhaps the most important of the *P. gingivalis* proteolytic enzymes, and if expressed *in vivo*, would be a major destructive enzyme (virulence factor) associated with the soft tissue destruction characteristic of human periodontitis. Several investigators (25, 190, 252) have postulated that the *P. gingivalis* collagenase may participate with host-derived collagenase in the destruction of gingival collagen. Birkedal-Hansen et al. (31) and Uitto et al. (424), for example, postulated that a proteinase from *P. gingivalis* may directly induce production of collagenase from human gingival fibroblasts.

It was almost 35 years ago that Schultz-Haude & Scherp (354) demonstrated that a mixed culture of bacteria isolated from the gingival cavity displayed collagenolytic activity. This activity was demonstrated to be a collagenase from *Bacteroides melaninogenicus* (121). Several subsequent studies (30, 237, 395, 425) described collagenolytic activity in a large number of *Bacteroides* species. The activity was cell-associated and was enhanced when the strains were grown in peptide-depleted medium in the presence of [¹⁴C]-labeled collagen as substrate (340). Toda et al. (415) demonstrated that the addition of reducing agents to the collagen-containing growth medium significantly enhanced the collagenolytic activity of all of the *P. gingivalis* strains

tested and that the activation was specifically inhibited by thiol proteinase inhibitors, again suggesting that the *P. gingivalis* thiol-dependent collagenase was in fact a thiol-proteinase. Mayrand & Grenier (252) were able to dissect the collagenolytic activity into at least two activities: a specific collagenase and nonspecific proteinases. These thiol-dependent collagenolytic enzymes were purified from the spent culture supernatant, and their inhibition with serum components was studied (419). The enzyme had a molecular weight of 70 kDa (394). The spent growth supernatant also contained a soluble trypsin-like enzyme and one associated with the outer membrane vesicles. The purified enzyme had a molecular weight of 58 kDa and was capable of degrading human plasma fibronectin and native type I rat tail collagen. While many *P. gingivalis* strains produced cell-associated collagenolytic activity capable of dissolving reconstituted collagen (type I) fibrils and of cleaving the helical domain of types I, II and III collagens, the cleavage patterns were clearly distinct from the characteristic cleavage pattern of vertebrate collagenases (30). Therefore, it is not clear as to which collagenase (bacterial or host) is responsible for the *in vivo* destruction of collagen.

In contrast to the studies of Toda et al. (415) and Mayrand et al. (254), Smalley et al. (385) found that the collagenolytic activity was attenuated with dithiothreitol. Comparison of the collagenolytic activity found in the extracellular vesicle fractions from virulent W50 and an avirulent colonial variant W50/BE1 showed that both strains depolymerized collagen producing identical polypeptide digestion patterns. The degradation yielded approximately 90% and 5% substrate degradation, respectively, similar to that found in whole cells of *P. gingivalis* W50 (386). The purified *P. gingivalis* collagenase was a protein of molecular weight 94 kDa which undergoes spontaneous cleavage to polypeptides of 75, 56 and 19 kDa. The enzyme was activated by reducing agents and had the capacity to cleave human basement membrane type IV collagen and synthetic collagen peptides (224).

In a very interesting study, Hoover & Felton (167) and Li et al. (232) used specific *P. gingivalis* collagenase-deficient mutants generated by nitrosoguanidine mutagenesis and showed that the mutants possessed significantly decreased interaction (that is, adherence) to *A. viscosus* compared with its wild-type parent. Takahashi et al. (408) were able to isolate a *prtC* gene from *P. gingivalis* strain 53977, which expressed collagenase activity. While the enzyme encoded by this gene possessed collagenolytic activity,

it was an unusual enzyme in that it possessed no gelatinase activity and was capable of degrading soluble and reconstituted fibrillar type 1 collagen as well as heat-denatured collagen and azocoll (190).

Molecular analysis of *P. gingivalis* proteolytic enzymes. Molecular techniques, including gene cloning, and the construction of defined functional mutants have been used to determine whether, in fact, *P. gingivalis* synthesizes the large number of proteolytic enzymes described in Table 5. The past decade has witnessed the cloning of *P. gingivalis* genes encoding a variety of proteinases, aminopeptidases, collagenases and hemagglutinins.

Progulske-Fox et al. (329, 330), Dusek et al. (82, 83) and Lee et al. (228) were the first to report the cloning and expression in *S. typhimurium* and *E. coli* of

four hemagglutinin genes from *P. gingivalis* strain 381: *hagA* (330), *hagB* (81, 82, 329, 331), *hagC* (230) and *hagD* (231). All appear to be involved in *P. gingivalis* hemagglutination. Recently, *hagA*, *hagB* and *hagC* mutants have been constructed and partially characterized (229).

Several very interesting genetic studies have demonstrated that the hemagglutination events mediated by *P. gingivalis* might be due to the combined effects of at least three enzymes formed into a large protein complex. The Arg- and Lys-gingipains and several *P. gingivalis* adhesion proteins have been found to be complexed together to form a "hemagglutination complex", all of which are transcribed from the same gene (3, 21, 314, 335, 375). The complex is formed on a high-molecular-weight polypeptide that is eventually enzymatically cleaved to pro-

Table 5. Isolated and characterized *Porphyromonas gingivalis* strain proteinase genes

Strain	Gene	Predicted size of protein (kDa)	Description	References
381	<i>hagA</i> , <i>hagC</i>	43, 38	Hemagglutinin	230, 329, 330
	<i>pgiM</i>	*	Methylase	19
	<i>hag</i>	50	Hemagglutinin	83
	<i>hagD</i>	*	Hemagglutinin	231
	<i>rgp</i>	109	Arg-gingipain precursor	361
	<i>hagB</i>	49	Hemagglutinin	331
	<i>hagC</i>	*	Hemagglutinin	230
	381- <i>kgp</i>	218	Lys-gingipain (mature form)	300
	381- <i>rgpB</i>	*	RGP-1 cysteine protease	452
	381- <i>rgpA</i>	*	Arg-gingipain	417
33277	<i>sod</i>	*	Superoxide dismutase	278
	<i>cpGR</i>	49.2	Glutamate dehydrogenase	181
	<i>cpGR</i>	*	Gingivain	118
	<i>rgpA</i> , <i>rgpB</i>	*	Arg-gingipain	279
	ATCC 33277- <i>rgpB</i>	*	Arg-gingipain	276
53977	<i>sod</i>	*	Superoxide dismutase	52
	<i>prtC</i>	*	Collagenase	408
	<i>prtC</i>	35	Collagenase	190
	<i>prtT</i>	53.9	Trypsin-like protease	309
	<i>prtT</i>	98	Protease	242
	<i>prtT</i>	96-99	Cysteine protease, hemagglutinin	243
W83	<i>tpr</i>	64	Thiol protease	32
	<i>Tpr</i>	80	Thiol protease	313
	<i>Tpr</i>	90	Thiol protease	312
	<i>prTH</i>	97, 110 kDa precursor	Protease	97
	<i>gdh</i>	49	Glutamate dehydrogenase	118
33277, W50, W12, W83	<i>pgag1</i>	51	PgAg1	182
W50	<i>prtI</i>	*	Protease 1	2
	<i>prpR1</i>	50	Protease (polyprotein for Arg1)	3
	<i>prt*</i>	45-50	Arg-cysteine proteinase, hemagglutinin	203
	<i>mcmA</i> , <i>mcmB</i>	68, 78	Methylmalonyl-CoA mutase	178
	W50- <i>prtR</i>	45, 44, 15, 17, 27	Arg-specific proteinase, adhesin	375
	W50- <i>prpR1</i> , <i>prR2</i>	*	Arg-x proteases-R1IA, R1IB	334, 339
	W50- <i>tlA</i>	*	R1 protease precursor	5
	W50- <i>prR2</i>	*	R1IA and R1IB-protease	4
H66	<i>rgp1</i>	50	Arg-gingipain-1	315
H66, W50	H66, W50- <i>kgp</i>	60	Gingipain K (KGP)	314
W12	<i>prtP</i>	186	Porphyphain	21

*No reported data.

duce a functional proteinase from the *N*-terminal end of the molecule, and multiple adhesion sites from the *C*-terminal portion of the polyprotein. While the *P. gingivalis* strains used by the different research groups for the purification of the polyprotein genes were different, the various cloned proteins were, however, homogeneous (324, 420). All of the cloned proteins, with only one exception (21) encoded a cysteine proteinase with either arginine- or lysine-specificity.

Duncan et al. (78) have demonstrated that the *P. gingivalis* hemagglutinins may also participate in the binding of the bacterium to host cells other than red blood cells. These investigators were able to identify *E. coli* clones carrying *P. gingivalis* genes, which were capable of adhering to several epithelial cell lines. All of the cloned DNA contained regions of homology not only to *hagA* and *hagD* (the hemagglutinin genes from *P. gingivalis* (229) but also to a cysteine proteinase gene (21)). Du et al. (75) determined that the hemagglutinating adhesion protein, HA-Ag2 from *P. gingivalis* also participates in its adherence to epithelial cells. While HA-Ag2 and the fimbriae are separate proteins formed in separate structures, immunological data indicate that they share common epitopes and, while they are physically separate on the *P. gingivalis* surface, they are physically situated very close to each other (47). Most studies, however, have concluded that the fimbriae are not essential for *P. gingivalis*-mediated red blood cell hemagglutination (137, 246). This latter observation is contrary to the results of Ogawa & Hamada (290) and Ogawa et al. (293), who used purified *P. gingivalis* fimbriae and synthetic peptides based upon the amino acid sequence of the mature fimbriae and demonstrated that the *P. gingivalis* fimbriae do play a direct role in hemagglutination. While it remains to be clarified whether *P. gingivalis* fimbriae participate in hemagglutination, it is clear that their synthesis, secretion and assembly are in some way interrelated with the hemagglutinins and cysteine proteinases (46).

Arnott et al. (16) identified a proteinase gene encoding for a thiol-dependent thiol proteinase. This enzyme had a molecular weight of 90 kDa and has been cloned and characterized (313). These investigators have recently constructed a thiol-deficient isogenic mutant from *P. gingivalis* strain W83 that lacked the *tpr* gene (*Tpr*⁻). The mutant phenotype expressed was that of a significantly reduced ability to hydrolyze collagen.

A *P. gingivalis* gene encoding a glycypropyl

aminopeptidase was cloned by Nakamura et al. (275); however, there have been few studies of this gene to date. In contrast, Kuramitsu's group (190, 408) have described a collagenase gene, *prtC*, that expresses collagenase activity as well as a *prtT* gene that encodes a protein expressing trypsin-like proteolytic activity (309). The *prtT* gene was larger than the *prtC* gene and encompassed the region that codes for *P. gingivalis* hemagglutination activity. The expressed proteins, 96 to 99 kDa, had significant homology to two streptococcal proteinases (242, 243). In another study, Fletcher et al. (97) isolated the gene *prtH*, which they found to express a 97-kDa protein that hydrolyzed human C3 complement and casein. This gene was similar to, if not identical to the gingipain gene (*rgp1*).

It is only in recent years that the genes responsible for the expression of the cysteine proteinases, the gingipains, have been cloned and characterized. Pavloff et al. (315), isolated and cloned the *rgp-1* gene, which encoded a 50-kDa cysteine proteinase which is now known as gingipain or argingipain-1 (RGP-1). The complete sequence of the gene revealed it to be unique among the cysteine proteinases. This protein was found to be synthesized initially as a polyprotein encoding a proteinase domain and four adhesin domains. Pavloff et al. (314) recently reported the complete sequence of the 60 kDa Lys-gingipain (KGP-1) gene, which is very similar to RGP-1 in its structural organization, biosynthesis and maturation.

Okamoto et al. (301) described for the first time the cloning and sequencing of the gene for the RGP cysteine proteinase, argingipain from *P. gingivalis* strain 381. The molecular weight of the extracellular mature enzyme was 44 kDa, but the nucleotide sequence of the isolated gene revealed a single gene coding for a 109-kDa precursor of argingipain. This precursor contained four functional domains: the *N*-terminal signal peptide domain required for cytoplasmic (inner) membrane transport; the *N*-terminal prosequence required to stabilize the precursor structure; the proteinase domain; and the *C*-terminal hemagglutinin domain, which appears to be essential for extracellular secretion of the proteinase domain. Nakayama et al. (279) have constructed arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants (*rgpA* and *rgpB* single and double mutants) and characterized their function. Both mutants were devoid of proteolytic activity, were not capable of inhibiting polymorphonuclear leukocyte function and were only able to initiate minimal hemagglutination. Interestingly, the double mutant was

also either devoid of fimbriae or possessed very few on its surface. The mutant also produced small amounts of two immunoreactive proteins (45 and 43 kDa), which corresponded to the precursor and mature forms of fimbriillin, respectively (280). Similarly, the Lys-gingipain encoding gene *kgp* has been cloned from *P. gingivalis*, and its complete nucleotide sequence has been determined with a calculated molecular mass of 218 kDa. This precursor of KGP was found to comprise at least four domains, the signal peptide, the *N'*-terminal prodomain, the mature proteinase domain, and the *C'*-terminal hemagglutinin domain. It was proteolytically processed during its transport (300, 349). These investigators further cloned and sequenced the second *rgp* gene (*rgpB*). A comparison of the two Arg-gingipain-encoding *rgp* genes revealed that their gene structures were very similar to each other, except that the *rgpB* gene did not possess most of the hemagglutinin domain present in the *C'*-terminal region of the *rgpI* gene (one of the *rgpA*-equivalent genes) and provided strong evidence for homologous recombination between the proteinase domain regions of the two *rgp* genes (276) (see section on genetic recombination).

The complete nucleotide sequence of the *P. gingivalis* gene *prtR*, which encodes a 132 kDa protein that contains an arginine-specific (45-kDa) thiol endopeptidase domain, as well as a hemagglutination domain, were cloned and characterized (203). The *prtR* nucleotide sequence encodes a polyprotein containing the Arg-specific proteinase and multiple hemagglutinins and adhesins. The nascent polyprotein consists of a putative leader sequence and a prosequence followed by the 45 kDa Arg-specific proteinase and 44-, 15-, 17- and 27-kDa sequence-related adhesins in that order (375). Following the previous demonstration that one of the major extracellular antigens of *P. gingivalis*, a 47-kDa protease, elicits an elevated serum IgG antibody response in adult periodontitis patients (60, 61), the gene encoding protease I was cloned and its functions characterized (2). The gene for ArgI (*prpR1* [protease polyprotein for ArgI]) was also cloned and sequenced. *prpR1* encodes three distinct extracellular Arg-x proteases (RI, RIA and RIB), organized into four distinct domains (pro, α , β and γ) and is processed to a heterodimeric protease (RI) that comprises the α -component (protease active site) and β -components in a noncovalent association (3). DNA sequences homologous to the coding region for the RI β -component are present at multiple loci on the *P. gingivalis*

chromosome, and further cloning, sequence analysis, and characterization of one of these homologous loci identified a gene similar to TonB-linked receptors (*tla* and TonB-linked adhesin), which are frequently involved in periplasmic translocation of hemin, iron, colicins or vitamin B₁₂ in other bacteria. An isogenic mutant was unable to grow in low hemin medium and produced significantly less arginine- and lysine-specific protease activities than the parent strain, indicating a regulatory relationship between *tla* and other members of this gene family (5). Furthermore, the distribution of *prpR1* and its variation within 43 isolates of *P. gingivalis* was determined. The presence of a second locus (*prR2*) homologous to the α region of *prpR1* was also detected, and these results suggested that the *prpR1* and *prR2* loci are maintained in natural populations of *P. gingivalis*, and only minor polymorphisms were detectable within the catalytic domain (8). Inactivation of the *prpR1* gene from isogenic mutants enabled the detection in the culture supernatant of two related Arg-x proteases (RIA and RIB). These two latter proteinases were identical to RIA and RIB, obtained by cloning and sequencing the *prR2*, a second genomic locus (335). Subsequently, the *prpR2* gene was insertionally inactivated and the mutant exhibited reduced total Arg-X activity, specifically the monomeric enzymes derived from *prpR1*, while the heterodimeric enzyme, R1, was unaffected by this mutation. The monomeric enzyme was radically different from the behavior of RIA and RIB from the parent strain. These data therefore suggest that the product(s) of *prp2* plays a significant role in the maturation pathway of *prpR1*-derived enzymes (4).

The gene encoding porphypain (*prtP*), a 186-kDa cysteine proteinase, was cloned and sequenced. It either has two separate active sites or one active site that can assume different conformations. However, while the data support the presence of at least these two active sites, this proteinase appears to be similar to RGP-1 and RGP-2. The *prtP* protein had regions of high homology to *hagA*, a hemagglutinin of *P. gingivalis*, and to several purported proteinases that have Arg-X specificity (21).

In addition to the *P. gingivalis* proteinase genes cloned and characterized above, other proteinase genes that have been identified include the gene encoding protease 1 (2), the gene for the synthesis of ArgI (*prpR1*) (3). Recently, a major cell-associated proteinase-adhesin complex designated PrtR-PrtK, composed of Arg- and Lys-specific cysteine proteinases and sequence-related adhesins (300 kDa) have

been found to be encoded by two genes, *priR* and *priK* (27)

Formation and regulation of selected enzymes from *P. gingivalis*. In addition to the large number of proteinases already identified in *P. gingivalis* strains, high levels of several other important biosynthetic and degradative enzymes have been identified (80, 195, 197, 222, 223, 378, 413). Alkaline phosphatase has, for example, been found in high levels in all of the *P. gingivalis* strains so far examined. While the enzyme appears to be cell-associated and limited to the periplasmic space, some evidence indicates that some of the enzyme is located in the extracellular environment. However, it is more than likely that this extracellular location is a result of either cell lysis or extrusion of outer membrane vesicles into this milieu. The *P. gingivalis* alkaline phosphatase is formed as a homodimer with a molecular weight of 61 kDa and 130 kDa, the latter apparently the molecular weight of the native enzyme. It is involved in the hydrolysis of casein and *o*-phosphoserine, suggesting that alkaline phosphatase can function as a phosphoprotein phosphatase (450). Alkaline phosphatase activity has been shown to be positively correlated with periodontal disease activity (29) as well as with alveolar bone resorption in advanced cases of human periodontitis (100). However, the role of alkaline phosphatase in *P. gingivalis* pathogenesis has not been clearly defined.

The ability of *P. gingivalis* to detoxify the potentially destructive superoxide radical was investigated by Amano et al. (10, 11). While oxygen is essential for the growth and metabolism of many prokaryotes, it is toxic to the bacterial species that grow and live under reduced and anoxygenic conditions. While oxygen possesses this beneficial quality, the molecule is very toxic to anaerobic species because of its ability to form highly reactive chemical species. For the most part also, mammalian cytochrome and other proteins do not reduce O_2 to the superoxide radical and are therefore protected from the toxic effects of this anion. However, in some cases, this anion is formed from, for example, the oxidation of Fe^{+2} of hemoglobin to Fe^{+3} . The protonation of the superoxide anion produces a hydroperoxyl radical (HO_2) capable of spontaneously reacting with other superoxide anions to form H_2O_2 . The superoxide anion is scavenged by superoxide dismutase, which functions in the conversion of two superoxide anions into H_2O_2 and O_2 . Under the catalytic direction of catalase, H_2O_2 is converted to O_2 and H_2O . While *P. gingi-*

valis, by definition, is an obligate anaerobe, it can withstand significant amounts of oxygen (Kesavalu et al., unpublished). Therefore, *P. gingivalis* is an anaerobe and cannot grow in an aerobic environment but can tolerate high levels of dissolved oxygen, it must possess the enzymes necessary for detoxifying oxygen radicals. These enzymes, superoxide dismutase, peroxidase and catalase, are capable of providing this protection. Superoxide dismutase was isolated and purified from *P. gingivalis* (10) and its amino acid sequence determined (11, 277). Superoxide dismutase-negative mutants were unable to grow in the presence of even small amounts of oxygen, while wild-type strains were aerotolerant. Since *P. gingivalis* is found as a member of the commensal microbiota, emerging and declining as a function of disease activity, and since it has been observed to be invasive, living under tissue oxidative stress, its ability to produce superoxide dismutase provides it with the mechanism(s) to withstand oxygen-generated radicals. The presence of superoxide dismutase also might be important to its ability to resist superoxide generation by polymorphonuclear leukocytes.

Slomiany et al. (377) also demonstrated that *P. gingivalis* elaborates an extracellular sulfatase capable of removing the sulfate ester groups from both sulfated glycosphingolipids (the structural constituents of the cell membrane of gingival epithelium) and from the glycosaminoglycan component of gingival connective tissue proteoglycans. The purified sulfatase (37 kDa) exhibited maximum glycosulfatase activity at pH 5.

In addition to other enzymes, *P. gingivalis* also possesses heparinase (200 and 150 kDa) and chondroitinase (70 kDa). These enzymes were extracted and purified from the vesicle fraction by a differential centrifugation technique and were capable of degrading gingival proteoglycans such as chondroitin-4-sulfate and heparin sulfate (387).

Banas et al. (19), reported the nucleotide sequence of a new methylase gene, *pgiM*, cloned from *P. gingivalis*. The gene shared significant homology with the methylases from *Streptococcus pneumoniae* and *E. coli*. A methylmalonyl-CoA mutase gene (178) and a 49-kDa NAD-dependent glutamate dehydrogenase gene (118, 181, 182) have been cloned, sequenced and characterized.

In summary, the enzymatic activities of *P. gingivalis* more than likely are important virulence factors in the interaction of *P. gingivalis* in the murine model, and possibly in the confines of the periodontal niche. The trypsin enzyme (Arg-, Lys-gingi-

pain), if active in a human host, could enhance the invasion of host cells and the spread of the bacterium and its secreted enzymes (that is, outer membrane vesicles) into the deeper tissues of the periodontium. Therefore, while the role of *P. gingivalis* enzymes in human periodontitis remains to be clearly determined, the results to date suggest very strongly that there is an important relationship between hemin (iron) uptake, pigmentation, enzymatic activity (trypsin, collagenase) and hemagglutination and hemolytic activity. This is further supported by recent genetic evidence linking the expression of the genes encoding these various factors, which could act in a synergistic way to effect soft tissue lesion formation in the murine model, and in the periodontal environment could function in a similar fashion.

Environmental regulation of outer membrane protein expression

Environmental cues and virulence factor gene expression

In order to survive in their respective host, microorganisms must sense subtle changes in their environment and adapt accordingly. Microorganisms typically adapt to environmental cues by "turning on and off" the expression of virulence genes which, in turn, allows for optimal growth and survival in different environmental niches. This adaptation strategy includes sensing and responding to changes in nutrients, pH, temperature, oxygen tension, redox potential, microbial flora and osmolarity (259). The ability to quickly adapt to a new environment often determines the rate of colonization, proliferation and, especially for human pathogens, successful infection. The adaptation process typically involves first sensing these environmental changes and then responding by expressing gene products that function to enhance establishment and survival *in vivo*. Regulation of virulence genes in response to environmental cues is undoubtedly an important step in the establishment of a pathogen. It is critical that genes be expressed during certain periods of infection. In general, virulence factors are not necessarily essential structural components or essential functions of cells, although "virulence factors", in a broad sense, have been used to define any bacterial factors that confer upon a bacterium the ability to adhere to, penetrate, replicate and colonize host cells. Indeed, many virulence factors are required only after introduction into a specific niche in the host, where they

are required for the establishment of a successful infection. Thus, throughout the different stages of infection, different sets of virulence factors are turned on and off in responses to different environmental cues (259). This allows a bacterium to adapt to its varying environment effectively.

Environmental cues in periodontal disease

During the transition from health to disease, a number of environmental changes are apparent in the oral ecological niche occupied by *P. gingivalis*. We speculate that these environmental changes serve as important cues for these organisms to vary gene expression. The gingival sulcus and periodontal pockets do not represent favorable environments for oral microbiota to proliferate and colonize, and only limited numbers of highly evolved species are capable of colonizing in this area (389). This is assumed to result from changes in the pH and temperature and in the availability of nutrients and oxygen which can limit the growth of bacteria. The mean temperature of the gingival sulcus during health is 35°C, with a consistent range of 30° to 38°C (388). The pH range within gingival pockets during health has been reported to be between 7.0 to 8.5 (55). It has been observed that, as disease progresses, the pH in the periodontal pocket increases as the periodontal pockets deepen and the host inflammatory response is induced (28). At this point the microbiota shifts from a predominantly gram-positive facultative to a predominantly gram-negative anaerobic one (63, 249). The optimal pH for growth of *P. gingivalis* is within the range of 7.5 to 8.0 (249); however, *P. gingivalis* has often been recovered from disease sites with elevated pH. The ability of *P. gingivalis* to grow at elevated pH may confer a significant advantage to this bacterium over other species and may explain its association with disease. It is likely that such extremes in the oxidation-reduction potential could be a limiting factor for certain species of microorganisms (388).

Nutrients are limited in the subgingival crevice, and successful periodontal pathogens must be capable of capturing essential nutrients for growth (389). Perhaps the most obvious of these nutrient requirements is the requirement of *P. gingivalis* for hemin (249). Although hemin is a major component of gingival crevicular fluid, host iron-chelating proteins such as haptoglobin, albumin and hemopexin make hemin unavailable for microorganisms (84, 316). *P. gingivalis* has evolved a number of mechanisms to capture iron and hemin for growth (see below).

Interactions between pathogenic and commensal species may influence colonization by *P. gingivalis*. Some species may produce growth factors or nutrients beneficial to the attachment and growth of *P. gingivalis* or may produce antagonistic materials that challenge the survival of *P. gingivalis*. Socransky & Haffajee (389) observed that black-pigmented anaerobic rods, including *P. gingivalis*, could benefit from other β -hemolytic species such as *Actinomyces* spp. and *Prevotella* spp. The presence of *Capnocytophaga ochracea* has been observed to correlate with a decrease in the risk for new attachment loss in the diseased person, indicating that this organism may serve as an antagonistic for *P. gingivalis* growth (389).

During the early onset of periodontal disease, polymorphonuclear leukocyte infiltration within the subepithelial connective tissue and the loss of epithelial cell attachment to the tooth enamel are observed. Subsequently, T cells and macrophages are recruited into the area underlying the pocket epithelial cells. This forms an array of antagonistic cells with release of biologically active cytokines such as IL-1, IL-6 and tumor necrosis factor- α (63). However, *P. gingivalis* has developed sophisticated mechanisms to evade or overcome the host defenses. Thus, for example in response to the host inflammatory response, *P. gingivalis* produces an extracellular capsule that inhibits phagocytosis (304).

Adaptation strategies of *P. gingivalis* in response to environmental cues

Temperature. Like other pathogenic microorganisms, an important homeostatic mechanism that *P. gingivalis* mounts when exposed to elevated temperature is a heat shock response (240, 429). This response is a highly conserved response to elevated temperature found in both prokaryotes and eukaryotes. Heat shock proteins function as molecular chaperones and are involved in protein folding and oligomerization of structural proteins (117, 265) and DNA replication (464). GroEL (HSP60 family) and DnaK (HSP70 family) homologs have been described in *P. gingivalis* (240, 429). In addition to the response to temperature changes, Vayssier et al. (429), have also reported that GroEL is induced by an increase in oxygen tension and by acidic pH. Amano et al. (9) have reported on a heat shock-inducible 55-kDa protein that may be related to HSP60. Further studies are needed to clarify the exact types and numbers of heat shock proteins synthesized by *P. gingivalis* in response to an upward shift in temperature and to determine the function of these heat

shock proteins and their involvement in pathogenicity.

Amano et al. (9) have also reported on the differential expression of *P. gingivalis* fimbrillin and superoxide dismutase in response to changes in temperature. These authors found that fimbrillin expression decreased in response to growth temperature. In a separate study, *P. gingivalis* was observed to exhibit an 11-fold increase in the fimbrillin (*fimA*) promoter activity when the growth temperature was shifted from 37°C to 34°C (446). Although the precise biological function of temperature-regulated fimbrillin expression and superoxide dismutase activity is not clear, it is suggested that downregulation of fimbrillin expression may help to evade the host immune system and that elevated superoxide dismutase activity may enable *P. gingivalis* to survive in environments containing high levels of superoxide produced from neutrophils (9).

pH. Stable growth of *P. gingivalis* is established in the range of pH 7.5 to 8.5, with optimal growth at pH 7.5. Trypsin-like activity has been observed to increase with increasing pH, with optimum activity between pH 7.0 and 8.0 (126, 256). Similarly, chymotrypsin-like activity was demonstrated to be maximized between pH 6.7 and 8.3 (256). The ratio of trypsin-like activity to collagenolytic activity at neutrality is 1:1, but rises to 7:1 at pH 8.3, suggesting that, during initial stages of infection, *P. gingivalis* responds to the environmental pH changes by producing enzymes with maximal potential for damaging host tissue. The majority of trypsin-like activity produced by *P. gingivalis* is due to the production of the arginine- and lysine-specific cysteine proteinases encoded by gingipains R and gingipain K, respectively. Optimal activity of gingipain R2 is pH 7.5–9.0 and that of gingipain R1 is pH 7.5–8.5. The increased activity of the *P. gingivalis* gingipains at higher pH values may serve to inactivate host immune system components such as complement and immunoglobulins (249, 256).

Oxygen. Hanioka et al. (144) have found that an increase in the hemoglobin concentration and a decrease in the oxygen saturation of hemoglobin were associated with the progression of gingival inflammation in human subjects. These results indicate that, although there is an increase in blood supply, it may not be sufficient to supply the needed oxygen in the inflamed gingiva. Detection of stress proteins in *P. gingivalis* by Vayssier et al. (429) indicated that an increase in oxygen concentration can induce the

HSP60-like stress protein (see above). However, little is known regarding the expression of *P. gingivalis* genes in response to oxygen.

Iron and hemin. Growth of a pathogen within a particular environmental niche depends in part upon the ability of a pathogen to scavenge essential nutrients. Of these nutrients, iron plays a crucial role in the establishment and progression of an infection (443). Within the human host, the majority of iron is found intracellularly; much is in the form of hemoglobin, heme proteins or ferritin. The small quantities of extracellular iron are complexed to carrier proteins including transferrin found in serum and lactoferrin present on mucosal surfaces (443). Due to their abundance in the host, heme-containing compounds are potentially a valuable source of iron for invading microorganisms. Pathogens that occupy intracellular niches *in vivo* can utilize heme directly. However, for extracellular pathogenic bacteria heme must first be released from the cell. This typically occurs by some form of tissue damage resulting in the release of intracellular material. Heme and hemoglobin are bound by the plasma proteins, haptoglobin and hemopexin. Microorganisms must possess mechanisms to remove the heme moiety or inorganic iron from such host iron-binding proteins.

The ability to utilize heme and heme-containing compounds for nutritional iron has been documented for several pathogenic bacteria (311), including *P. gingivalis*. Several studies (37, 311) have shown that *P. gingivalis* is capable of utilizing *in vitro* a broad range of heme containing compounds such as hemoglobin, myoglobin, hemopexin, methemoglobin, oxyhemoglobin and cytochrome C. *P. gingivalis* can also grow with non-heme iron sources, including ferric and ferrous inorganic iron, and human transferrin.

P. gingivalis may also be capable of storing heme on its cell surface; the characteristic pigmentation produced by *P. gingivalis* colonies appears to be due to the accumulation of heme and has been postulated to serve as a mechanism for heme storage (357). The ability of *P. gingivalis* to store heme appears to provide a nutritional advantage for the survival of this pathogen in the iron-limited environment of the healthy periodontal pocket. The environmental conditions in the periodontal pocket during the course of an infection are not precisely known; however, high numbers of erythrocytes are typically found in diseased sites (267). *P. gingivalis* possesses a hemagglutinin, hemolysin and several enzymatic activities that may promote colonization

by aiding in the acquisition of heme or iron (53, 253). The *P. gingivalis* hemolysin may function to lyse erythrocytes *in vivo*, resulting in the liberation of heme. *P. gingivalis* also appears to be highly effective at degrading the plasma proteins, albumin, hemopexin, haptoglobin and transferrin (253).

Mechanisms of heme and iron transport in *P. gingivalis*. Microbial assimilation of host iron can occur by several different mechanisms (443). One such mechanism involves the production of siderophores, low-molecular-weight compounds with an extremely high affinity for ferric iron. Another mechanism involves the production of iron-regulated proteins, which function to acquire iron directly from the host iron-binding proteins. In most gram-negative bacteria, iron transport is typically characterized by a specific transport system that involves iron-regulated outer membrane protein receptors, periplasmic binding proteins and cytoplasmic membrane proteins (443). Putative heme binding receptors have been identified in *Haemophilus influenzae*, *Haemophilus ducreyi*, *Vibrio cholerae*, *Yersinia enterocolitica*, *Neisseria meningitidis* and *Neisseria gonorrhoeae* (145, 148, 225, 396); and a heme storage protein has also been described in *Yersinia pestis* (18).

To date, siderophores have not been isolated from *P. gingivalis*. *P. gingivalis* does express a number of novel outer membrane proteins in response to iron and heme limitation (33); however, the specific role of these proteins in iron-acquisition has not been definitively defined. Genco et al. (111) have established that the heme molecule binds to the cell surface and is transported into the cell by a process that requires energy. Both protoporphyrin IX and heme can compete for radiolabeled heme binding and transport, indicating that an outer membrane receptor specific for the protoporphyrin IX ring is involved in heme binding. Heme binding under non-growing and growing conditions, as well as heme transport in *P. gingivalis*, are induced by increased levels of heme itself. It thus has been speculated that a heme-inducible component(s) is involved in heme binding and transport in *P. gingivalis*.

Induction of heme binding and transport in *P. gingivalis* is novel, and only a few reports have documented such a phenomenon (44, 382, 384). Smalley et al. (382) have reported that growth under heme-excess conditions resulted in increased heme-binding capacity of *P. gingivalis* extracellular vesicle preparations, whole cells, and EDTA-extracted outer membranes. Carman et al. (44) have also demonstrated that *P. gingivalis* whole cells and outer mem-

branes bind significant amounts of hemin when bacteria are grown in complex media supplemented with hemin. Taken together, these results suggest that in *P. gingivalis* hemin may induce the production of new outer membrane proteins that may function in hemin binding and transport and may represent a novel mechanism(s) for the acquisition of hemin.

In addition to hemin-inducible outer membrane proteins, a number of reports have described the production of hemin-repressible proteins in *P. gingivalis*. Bramanti & Holt (33, 36) have described a 26-kDa protein that is produced by *P. gingivalis* grown under hemin limitation and have proposed a role for this protein in hemin binding. This outer membrane protein appears to bind hemin when *P. gingivalis* cultures are grown in the presence of [⁵⁵Fe]-hemin.

Chu et al. (53) studied five *P. gingivalis* strains for their ability to lyse red blood cells, and all five strains produced a functional "hemolysin" associated with the outer membrane. While the hemolysin was produced throughout the growth cycle, significant amounts of the protein were formed during late exponential to stationary growth phase, with significant hemolytic activity being concentrated in the outer membrane vesicles. Functionally, the concentration of the hemolysin in the vesicles could provide the important mechanism for the bacterium to compete effectively for hemin in the confines of the periodontal pocket. The large number of vesicles formed during growth could function to "attack" and hemolyze red blood cells during periods of active disease. Karunakaran & Holt (188) cloned two *P. gingivalis* hemolysins, one of 48 kDa molecular weight, and the other 18 kDa.

Bramanti & Holt (35) have provided some of the first data indicating that the expression of at least ten surface proteins with molecular weights between 26 to 80 kDa was observed in *P. gingivalis* W50 grown under hemin-depleted conditions. Growth of *P. gingivalis* strain W50 in the presence of normal to excess hemin (hemin replete; required for growth of *P. gingivalis*) resulted in the downregulation of these proteins. Growth under conditions in which hemin was a limiting growth factor (hemin deplete) resulted in the expression of at least 12 outer membrane proteins not found in the outer membrane of cells grown in hemin replete conditions. Two of these "hemin-regulated outer membrane proteins" at approximately 83 kDa and 26 kDa, were recognized as major hemin-regulated *P. gingivalis* W50 outer membrane proteins. Bramanti & Holt (33) postulated that the hemin repression observed required the pres-

ence of protoporphyrin IX. Growth of the hemin-depleted cultures in normal or hemin-replete medium resulted in the rapid repression of the OMP26 (as well as the other hemin-regulated outer membrane proteins). However, OMP26 was not repressed in the presence of lactoferrin, transferrin, inorganic iron, zinc protoporphyrin IX or protoporphyrin IX. Radioiodination studies revealed that OMP26 was no longer accessible to iodine labeling after less than one minute of culture shift from hemin-deplete to hemin-replete culture (36). SDS-PAGE analysis revealed that OMP26 migrated with an apparent molecular weight of 39 kDa in its unheated closed molecular configuration. High-resolution immunogold electron microscopy confirmed the rapid migration of OMP39 into the outer membrane, where it was no longer surface accessible to both immunogold labeling and radioiodination (36). Therefore, OMP26 might function to bind hemin under hemin-depleted conditions and transport it across the outer membrane and into the cytoplasmic region where it would be used in a variety of anabolic reactions. Bramanti & Holt (38) also reported that hemin-starved cells bound much higher amounts of [⁵⁵Fe]-hemin than cells grown in heme-rich conditions. Purified monospecific polyclonal anti-26 kDa antibody was capable of inhibiting hemin binding to hemin-starved cells. The binding of the hemin to the cells was also determined to be mediated by the protoporphyrin IX molecule since unlabeled hemin, protoporphyrin IX, zinc protoporphyrin and Congo red inhibited [⁵⁵Fe]-hemin binding but non-hemin iron sources such as transferrin and lactoferrin did not inhibit hemin binding. Direct binding of hemin to OMP26 was observed using a photoreactive cross-linking reagent. Recently, Kim et al. (200) isolated, purified and biochemically characterized a 30-kDa (heated=24 kDa) hemin-regulated hemin-binding protein from *P. gingivalis* strain 381. This protein was very similar to OMP26 from strain W50 and is more than likely its homologue in strain W50. *N*-terminal amino acid sequence analysis of the purified protein revealed no known homology with other hemin-binding classes of proteins.

Smalley et al. (384) have also reported on the identification of hemin-repressible proteins in *P. gingivalis*. These proteins were shown to bind hemin as detected by tetramethylbenzidine and Coomassie blue staining. These investigators have postulated that these proteins may represent a binding system for hemin uptake or processing that is induced only under low environmental levels of hemin. In this regard, Smalley et al. (383) have shown that binding

of ferriprotoporphyrin IX by *P. gingivalis* may occur through both high- and low-affinity binding sites.

The storage of hemin on the cell surface may be mediated by specific hemin storage proteins, as has been described in *Y. pestis* (317), or may involve common hemin-binding proteins. The mechanisms involved in the removal of iron from the hemin molecule are not known; however, it has been postulated that iron is removed from the hemin molecule within the periplasm. At this point, free iron interacts with a periplasmic binding protein, followed by transport of iron across the cytoplasmic membrane by a cytoplasmic permease; these steps are analogous to the well-described active transport processes in other gram-negative organisms (323). A putative *P. gingivalis* TonB-like protein would function to translocate energy from the cytoplasmic membrane to the outer membrane during the iron transport process. However, the specific *P. gingivalis* molecules participating in each of these processes have not been identified.

Under conditions of hemin limitation, hemin may be transported by a mechanism in which low-affinity receptors are expressed. Thus, binding of hemin in *P. gingivalis* may occur through both high- and low-affinity binding sites has been suggested by Smalley et al. (382). Hemin-repressible proteins produced by *P. gingivalis* may represent low-affinity binding receptors and may be components of a hemin-uptake system that is induced only under low environmental levels of hemin. In contrast, the hemin-inducible protein may be involved in high-affinity binding of hemin. Taken together, these results indicate that, in *P. gingivalis*, the expression of proteins involved in hemin transport may be positively and negatively regulated by hemin.

Recent communications have reported on the identification of outer membrane receptors that may be involved in hemin acquisition by *P. gingivalis*. Karunakaran et al. (189) have identified a gene encoding for a TonB-dependent outer membrane (*hemR*) and have postulated a role for this gene in iron transport. Transcription of *hemR* was demonstrated to be repressed under hemin replete conditions. To date, however, the precise role of *hemR* in hemin acquisition has not been elucidated. A gene (*hmuA*) closely related to the *P. gingivalis hemR* gene was identified recently (372). This gene shares 50% homology with *hemR* and 25–30% homology with a number of TonB-dependent outer membrane receptors involved in hemin and siderophore transport in other microorganisms.

The *hmuA* gene was highly homologous at the 5'

end with the hemin-regulated gene *hemR*; however, homology between the *hmuA* and *hemR* was greatly reduced at the 3' region. Karunakaran et al. (189) have shown that the 3' region of *hemR* exhibits identity to the *priT* gene of *P. gingivalis*. This homology of the –COOH-terminus of *P. gingivalis hemR* to *priT* suggests the possibility of two occurrences: 1) the identity that the C-terminus of *hemR* with *priT* may result from a rearrangement event that mediated the insertion of a portion of *priT* into *hemR* via homologous recombination, and *hmuA* is representative of the ancestral gene, or 2) the absence of homology between *priT* and *hmuA* may result from a rearrangement event in which this region was deleted from *hmuA*.

Karunakaran et al. (189) also identified another putative gene ORF1, between *hemR* and *priT*. The deduced amino acid sequence of ORF1 corresponded to a 48-kDa protein. Northern blots indicated that ORF1 was part of a 1.0-kb mRNA and was positively regulated by hemin levels. The *hemR* gene was part of a 3.0-kb polycistronic message and was negatively regulated at the transcriptional level by hemin. Primer extension analysis of the *hemR* gene revealed that the transcription start site was at a C residue located within ORF1. An examination of *HemR::lacZ* constructs in both *E. coli* and *P. gingivalis* confirmed hemin repression of *hemR* expression in both organisms. The *HemR* protein expressed in *E. coli* was detected by an antiserum from a periodontitis patient heavily colonized with *P. gingivalis* but not by serum from a periodontally healthy patient or by antisera against hemin-grown *P. gingivalis* cells. Therefore, it is likely that the 48-kDa *HemR* protein can be expressed only under hemin-restricted conditions.

The recently identified *tla* gene, which encodes for a TonB-dependent outer membrane receptor, is required for growth of *P. gingivalis* in low levels of hemin (5). Thus, *hemR*, *hmuA* and *tla* genes contain amino acid homology to the TonB box of iron-regulated genes from a variety of microorganisms. In *E. coli*, *Y. enterocolitica*, *S. typhimurium*, *H. influenzae* and *H. ducreyi* the TonB inner membrane protein provides energy for transport of ligands across the outer membrane (323). TonB spans the periplasmic space and physically interacts with outer membrane receptors in a highly conserved region called the TonB box. This interaction is thought to lead to a conformational change in the receptor protein, permitting transport of the ligands across the outer membrane into the periplasmic space. The TonB protein is required for a variety of energy-dependent

outer membrane processes, including uptake of all siderophore complexes, vitamin B₁₂ transport, the action of many colicins and the irreversible step in binding of bacteriophages such as T1 and 80. The TonB protein has recently been shown also to be required for the transport of hemin in *Y. enterocolitica* and *H. influenzae* (149, 396). The energy dependence of hemin transport in *P. gingivalis* suggests that a TonB analog anchored in the cytoplasmic membrane may also be present in *P. gingivalis* and function in the transport of hemin. Thus, it is probable that the TonB box of these *P. gingivalis* outer membrane receptors physically interacts with a yet unidentified *P. gingivalis* TonB protein.

Fujimura et al. (105, 106) were able to isolate a hemoglobin-binding protein from the outer membrane of *P. gingivalis*. The binding activity of this 19-kDa hemoglobin-binding protein was significantly higher in low pH buffers than at high pH, with optimum binding occurring at pH between 4.5 and 5.0. The binding was reversible at higher pH.

While the specific iron-binding proteins have not been completely identified, the binding kinetics of hemin, transferrin and hemoglobin appear to be very similar when *P. gingivalis* is grown in iron-limited conditions. Whether these observations support iron binding in *P. gingivalis* occurring via a single population of binding proteins or whether there are multiple binding sites remains to be determined. Bramanti et al. (37) studied the role of hemin on outer membrane protein expression and function in several *P. gingivalis* strains. Growth of these *P. gingivalis* strains in a variety of hemin-containing molecules (7.7 μ M equivalent of hemin as hemoglobin, methemoglobin, myoglobin, hemin-saturated serum albumin, lactoperoxidase, cytochrome C and catalase) resulted in similar growth rates and growth yields. Interestingly, growth of these strains in the presence of haptoglobin-hemoglobin and hemopexin-hemin complexes was identical to growth in the presence of equivalent amounts of hemin, indicating that these host defense proteins do not sequester hemin from *P. gingivalis*. Growth of *P. gingivalis* under hemin-depleted or hemin-free conditions or in the presence of inorganic iron resulted in the expression of a surface-exposed 26-kDa outer membrane-limited protein (38). [⁵⁵Fe]-hemin uptake studies and [¹²⁵I]-SASD-hemin cross-linking experiments revealed that OMP26 was in fact a hemin-binding protein in *P. gingivalis* W50 and functions as a key hemin transport process.

Bramanti & Holt (37) have also reported that the growth of *P. gingivalis* required an exogenous iron

source and that protoporphyrin IX did not support growth. Since *P. gingivalis* was capable of "good" growth when supplied with nonheme iron (ferric, ferrous and nitrogenous inorganic iron) as well as excellent growth in the presence of transferrin, lactoferrin, and serum albumin, it is more than likely that it is the iron atom that is essential for growth. However, the specific role of the iron-atom in *P. gingivalis* growth and survival needs to be determined. Hemin limitation also resulted in a significant increase in hemolysin production compared with *P. gingivalis* grown in the presence of excess or even normal growth levels of hemin (53). Heme-starved *P. gingivalis* also express at least two hemin-binding sites with affinities intermediate between those of serum albumin and hemopexin. Hemin-binding was reduced following treatment with protein-modifying agents (heat, pronase and *N*-bromosuccinimide) and was blocked by protoporphyrin IX and hemoglobin. Hemopexin also inhibited bacterial hemin binding (418).

Schifferle et al. (351) also examined the effect of protoporphyrin IX limitation on the growth and virulence of *P. gingivalis* strain A7A1-28. In medium varying between 0 and 5 μ M protoporphyrin IX, no effect on proteolytic enzyme production or virulence in a mouse abscess model was observed; however, protoporphyrin IX was supplied in limiting concentrations, there was a significant increase in the expression of five new outer membrane proteins and the suppression of at least four other outer membrane proteins. Shizukuishi et al. (369) reported that hemin, transferrin and hemoglobin supported comparable growth of *P. gingivalis* (growth rate and growth yields), while several inorganic iron compounds also supported growth, but at much lower rates and cell yields.

Many studies have demonstrated that lactoferrin has strong bacteriostatic and bactericidal effects as well as a role in regulating various components of the immune system. Some of these studies showed that lactoferrin binds readily to whole cells via high-affinity receptor, and all strains of *P. gingivalis* completely degraded lactoferrin by cell-associated proteases (69, 186, 204). However, very little is known concerning the specificity of these iron-binding proteins in *P. gingivalis*.

Purified lipopolysaccharide from both *P. gingivalis* normal (5 μ g of hemin per ml) and hemin-limited (0.08 μ g of hemin per ml) in a chemostat environment increased *N*-formylmethionyl-leucyl-phenylalanine-induced superoxide release by neutrophils in a dose-dependent manner. Hemin-normal gener-

ated lipopolysaccharide was a significantly more potent neutrophil priming agent than hemin-limited generated lipopolysaccharide. The differences in the biological activity of the two lipopolysaccharide preparations could be associated with structural differences, and these results indicate that hemin availability affects regulation of an aspect of *P. gingivalis* virulence, lipopolysaccharide-neutrophil priming (45).

A recent report demonstrated that *P. gingivalis* grown under hemin limitation did show almost a 50% decrease in β -galactosidase production (446) as well as the upregulation of several other important proteolytic enzymes (193). The activity of these proteolytic enzymes may be critical in the survival and growth of *P. gingivalis* in the periodontal pocket. Interestingly, the majority of these enzymes exhibited maximum enzymatic activities in alkaline pH 7 to 8, characteristic of the pH of the pocket during active microbial activity.

In addition to its role as an essential nutrient, iron serves as a regulatory signal in many organisms influencing the expression of a wide variety of bacterial proteins. In response to the low iron level found in the host, genes under negative control by iron are derepressed. These include not only iron transport systems necessary for iron acquisition but also toxins and other virulence factors (235). In addition to genes involved in hemin transport, there is growing evidence that hemin can influence the expression of putative *P. gingivalis* virulence genes. A number of conflicting reports have appeared in the literature describing both the induction and repression of expression of specific virulence factors in response to hemin. Interestingly, it has recently been documented that, in *Corynebacterium diphtheriae*, both iron and hemin can influence the expression of iron transport and virulence genes (353). In particular, it was shown that transcription of the *hmuO* gene, which is predicted to encode for a heme oxygenase, is activated by heme and repressed by iron (353). Thus, the conflicting reports with regard to the influence of hemin on the expression of *P. gingivalis* virulence factors may be due to differences in growth conditions used for these studies in which both iron and hemin were present.

The influence of hemin on the virulence of *P. gingivalis* has been documented by several investigators; however, the role of hemin in the regulation of specific virulence genes has not been precisely defined. Bramanti et al. (38) have demonstrated that hemin-restricted *P. gingivalis* cultures demonstrate greater lethality as assessed in a mouse model. In

contrast, several studies have reported that hemin limitation results in a decrease in the virulence of *P. gingivalis* (249, 257). The discrepancy in these results may reflect differences in media, growth conditions and animal models employed in these studies.

Growth of *P. gingivalis* under hemin limitation has been reported to result in a reduction in the number of fimbriae on the cell surface as well as in an apparent increase in the numbers of extracellular vesicles as detected by electron microscopy (257). Limitation of hemin during *P. gingivalis* growth has been reported to also affect hemolytic and hemagglutination activities of *P. gingivalis* whole-cell and extracellular vesicles. A 3.5-fold higher trypsin-like protease activity and a 2.5-fold decreased collagenolytic activity were found in hemin-restricted *P. gingivalis* cultures compared with the hemin-excess cultures (250). Vesicles isolated from hemin-limited *P. gingivalis* cultures have been shown to possess higher trypsin-like protease and hemagglutination activities (384). Interestingly, there is an increase in extracellular vesicle production by hemin-restricted *P. gingivalis* cultures (257), and although hemin-restricted cultures have a lower overall level of trypsin-like activity and a reduction in total culture biomass, the activity of extracellular vesicles from hemin-restricted cultures was three times greater than that of hemin-excess cultures (384). Analysis of *rgp2* transcription has recently revealed that *rgp2* transcription is decreased in response to hemin limitation (Potempa, personal communication). Since the majority of *P. gingivalis* strains examined in one study appear to produce gingipains R and gingipain K, it has been postulated that the involvement of these proteinases in virulence may be due to differential regulation and enhanced expression in virulent strains. However, definitive studies on the regulation of the gingipains by environmental factors have yet to be described.

Modification of the antigenicity of *P. gingivalis* lipopolysaccharide has also been reported in response to hemin limitation (62). A 26-kDa antigen (26 LPSC) refractory to Coomassie blue staining was lost upon transfer of *P. gingivalis* from hemin-excess into hemin-restricted conditions. Along with the loss of the 26 LPSC, a significant reduction in the hemin-binding capacity of *P. gingivalis* whole cells was observed (62). Western blot analysis using antisera raised to *P. gingivalis* A7436, W83 or HG864 grown in complex media (supplemented with hemin) indicated that antibody specific for the 26-kDa lipopolysaccharide epitope was also produced. The authors postulated that the hemin-induced lipopolysacchar-

ide moiety could serve as an outer membrane depot for hemin binding and storage during hemin-replete conditions.

Although these previous studies support the concept of the regulation of *P. gingivalis* virulence factors in response to hemin, evidence at the molecular level for hemin regulation has been lacking. Genco et al. (111) reported on transpositional insertion mutant of *P. gingivalis*, designated MSM-3, that was defective in hemin utilization and transport. *P. gingivalis* MSM-3, grown in either hemin-replete or hemin-depleted conditions, bound and transported less hemin and iron from hemin as compared with the wild type strain A7436. The defect in hemin binding by *P. gingivalis* MSM-3 was postulated to result from the inability to bind, store or remove hemin from the cell surface, resulting in the inefficient transport of hemin into the cell. The inability of *P. gingivalis* MSM-3 to efficiently transport hemin into the cell would render it in a constitutive hemin-limited state. Interestingly, many of the previously described characteristics of hemin-limited *P. gingivalis* cultures were constitutively expressed by *P. gingivalis* MSM-3. These include the hemolytic activity and trypsin-like proteolytic activity associated with *P. gingivalis* MSM-3 extracellular vesicles. *P. gingivalis* MSM-3 whole cells and vesicle preparations also exhibited a higher affinity for red blood cells as compared with the parent strain. *P. gingivalis* MSM-3 was also found to be more virulent in the mouse subcutaneous chamber model. The increased production of extracellular vesicles, the associated increase in proteolytic activity and hemagglutination of *P. gingivalis* MSM-3 were proposed to be responsible for the increased virulence of MSM-3 in the mouse subcutaneous chamber model. More recently, it has been determined that MSM-3 produces twice the *rgp2* transcript as compared to the parent strain (371). This may thus explain the increased virulence of this strain.

Iron as a regulatory signal. The molecular basis of coordinate regulation by iron has been most thoroughly studied in *E. coli*. In this organism, coordinate regulation of gene expression by iron depends on the ferric uptake regulator protein, Fur (150). Fur binds specifically to a Fur box, a 19-bp consensus sequence that overlaps the promoters of genes whose expression is iron-regulated. Fur acts as a classical negative repressor and uses Fe^{+2} as a corepressor to bind the promoter region of iron-regulated genes. At sufficient intracellular concentrations of iron, the Fur-iron complex binds to Fur-binding

sites and prevents transcription. Regulation of iron-regulated genes by a Fur-like system has been described in *Y. pestis*, *V. cholerae*, *Vibrio vulnificus*, *Pseudomonas aeruginosa*, *N. gonorrhoeae*, *N. meningitidis*, *H. influenzae* and *S. typhimurium* (26, 187, 235). These genetically diverse microorganisms thus appear to utilize similar mechanisms to control the expression of iron-regulated genes. It is interesting to speculate that, in response to hemin limitation, *P. gingivalis* is capable of turning on the expression of several factors that appear to be involved in the virulence potential of this organism. The results obtained by Genco et al. (114) support the concept of coordinate regulation of several putative *P. gingivalis* virulence genes by hemin. Regulation of hemin-responsive genes in *P. gingivalis* may occur by a negative regulator such as Fur. There is also the possibility that hemin and iron regulation in *P. gingivalis* may require more than one regulatory factor. In other organisms such as *V. cholerae*, *P. aeruginosa* and *S. typhimurium*, regulation of a number of virulence determinants by iron appears to be complex and requires more than one regulatory factor (236). One preliminary report indicates that *P. gingivalis* A7436 produces a Fur-like protein (116). Although previous studies support the concept that hemin limitation induces the expression of many *P. gingivalis* virulence factors, the mechanism of gene expression regulation has not been elucidated at the molecular level. Clearly, future studies should be aimed at examining the regulation of expression of iron-regulated genes in this pathogen.

Host response as cues for the induction of virulence factors

Growing evidence suggests that *P. gingivalis* has developed adaptation strategies to deal with the host immune system. The specific mechanisms involved in the initiation and continuation of a host destructive inflammatory response in periodontitis remain unknown. However, a general pattern appears to be emerging that demonstrates that the bacteria suspected of being associated with periodontal diseases have the ability to evolve mechanisms to evade or even inhibit the host immune defense system (65) and are more potent at inducing bone loss (272). For example, Loomer et al. (238) showed that sonic extracts obtained from bacteria associated with periodontitis (*P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia*) inhibited osteogenesis as compared with extracts from nonpathogenic bacteria. A recent report suggests that lipopolysaccharide iso-

lated from these periodontopathogens promotes osteoclast-like cell differentiation in bone marrow cell cultures (160).

Host cells of both myeloid and non-myeloid origin respond to bacterial lipopolysaccharide, thereby contributing to the initiation of an inflammatory response. Darveau et al. (66) found that *P. gingivalis* lipopolysaccharide, unlike lipopolysaccharide from other gram-negative bacteria, does not stimulate the expression of E-selectin by human endothelial cells and thereby hinders extravasation of leukocytes. Additionally, *P. gingivalis* lipopolysaccharide was shown to be a poor activator of the release of tumor necrosis factor- α and IL-1 β by blood mononuclear cells (66), most likely due to a lower capacity of *P. gingivalis* lipopolysaccharide to bind CD14 (59). Moreover, *P. gingivalis* was shown to inhibit the ability of other bacteria to stimulate E-selectin expression by endothelial cells (66). All these data are in agreement with the involvement of *P. gingivalis* in suppressing the host immune response (65).

The dense amorphous capsule surrounding *P. gingivalis* confers resistance to phagocytosis and to the bactericidal effects of serum and has been associated with an increased virulence potential in several animal models (404, 426). However, it is not known whether the synthesis of the polysaccharide capsule is constitutive *in vivo* or if it is induced in response to interactions of *P. gingivalis* to phagocytes. *P. gingivalis* proteinases have been reported to exhibit enzymatic activity against a broad range of host proteins (see the section on proteinases). The ability of *P. gingivalis* to control the expression of these proteinases in response to host inflammatory cells has, however, not been experimentally proven.

It is becoming increasingly evident that bacterial gene expression is quite complex and that bacteria may respond to particular signals induced *in vivo* and express genes required for *in vivo* growth. One report has recently described *P. gingivalis* gene expression *in vivo* using an *in vivo* expression technology system (228). This *in vivo* expression technology vector termed pPGIVET was constructed and used to determine whether the hemagglutinin genes *hagB* and *hagC* of *P. gingivalis* were expressed during an infectious process. The plasmid pPGIVET was constructed as a conjugative suicide plasmid containing a multiple-cloning site upstream of two tandem promoter-less reporter genes that encode tetracycline resistance and galactokinase. The promoter and a portion of the open reading frame of *hagB* were inserted into the multiple-cloning site in both a positive and a negative orientation relative to the

reporter genes. Southern blot analysis of different transconjugants indicated that Campbell insertions had occurred at the chromosomal *hagB* locus and also at the *hagC* locus, which has high (99%) homology to the open reading frame of *hagB*. pPGIVET-labeled clones in which the *hag* promoters were positively oriented relative to the reporter genes expressed tetracycline resistance and galactokinase activity *in vitro* and *in vivo* at significantly higher levels than did the wild-type strain or clones in which the *hag* promoters were negatively oriented. Expression of tetracycline resistance allowed substantial enrichment of heterodiploids over wild-type cells during a mixed infection in the mouse abscess model. These results indicate that *hagB* and *hagC* are transcriptionally active *in vivo* and suggested that pPGIVET may be used to isolate *P. gingivalis* genes expressed only during an infectious process. It is anticipated that additional virulence genes will be isolated by this technology.

It is well accepted that *P. gingivalis* virulence factors collectively contribute to pathogenesis. Researchers are now beginning to define how these virulence factors are regulated by environmental changes, and detailed molecular studies are beginning to emerge. It is apparent that this is an exciting era of research as it relates to the interactions of defined periodontal pathogens and the host. As seen with other microorganisms, the mechanisms of virulence gene regulation in response to environmental cues may be extremely complex within *P. gingivalis*. At the time of this writing, the *P. gingivalis* genome preliminary sequence has not been released. It is anticipated that, with the complete sequence in hand, additional bacterial virulence factors can be defined and the regulation of these factors can be examined in response to specific environmental cues. This may allow additional virulence genes to be identified and, most importantly, the components of the system(s) that regulate them to be determined. Understanding the mechanism of virulence gene regulation in response to the local environment of the host will provide crucial information in the development of effective treatments targeted at eradication of periodontal disease.

Invasion. Clinical studies have clearly demonstrated the ability of *P. gingivalis* to infiltrate the oral epithelium (341). This process is suggested to represent a highly adaptive approach to evading host defense systems. Adhesion of bacterial pathogens to the eucaryotic surface is mediated by macromolecules, known collectively as adhesins, that interact with re-

ceptors on the eukaryotic cells. Components on the surface of bacteria that are associated with adhesion include fimbriae, flagella, lipopolysaccharide, polysaccharide, capsule, microvesicles and outer membranes (286). Environmental factors influencing the expression of these bacterial adhesins will affect bacterial adhesion to host cell surfaces and, consequently, bacterial invasion of host gingival tissues. The importance of bacterial invasion of oral epithelium in periodontal diseases remains to be determined. However, host epithelial cells harboring *P. gingivalis* may provide a source of reinfection after mechanical debridement.

In vitro studies have confirmed bacterial adhesion to and invasion of gingival epithelial cells for *P. gingivalis* (79). Several *P. gingivalis* strains immobilized on filters were observed to bind oral epithelial cells. The invasion of epithelial cells by *P. gingivalis* ATCC 33277 was also confirmed by transmission electron microscopy (79). The adhesive and invasive potential of *P. gingivalis* interacting with human pocket epithelium and KB cell line *in vitro* was examined by Sandros et al. (344). Pocket epithelial tissue, obtained during periodontal surgery of patients with advanced periodontal disease, generated a stratified epithelium in culture. *P. gingivalis* laboratory strains and clinical isolates were tested with respect to epithelial adhesion and invasion. Adhesion was quantified by scintillation spectrometry after incubation of radiolabeled bacteria with epithelial cells. The invasive ability of *P. gingivalis* was measured by means of an antibiotic protection assay. All tested *P. gingivalis* strains adhered to and entered pocket epithelial cells. However, considerable variation in their adhesive and invasive potential was observed. Transmission electron microscopy revealed that internalization of *P. gingivalis* was preceded by formation of microvilli and coated pits on the epithelial cell surfaces. Intracellular bacteria were most frequently surrounded by endosomal membranes; however, bacteria devoid of such membranes were also seen. Release of outer membrane vesicles (blebs) by internalized *P. gingivalis* was observed.

Initial observations of invasion of primary cultures of gingival epithelial cells by *P. gingivalis* were reported by Lamont et al. (218). Optimal invasion occurred at a relatively low multiplicity of infection (100) and demonstrated saturation at a higher multiplicity of infection. Following the lag phase, during which bacteria invaded poorly, invasion was independent of growth phase. *P. gingivalis* was capable of replicating within the epithelial cells. Invasion was an active process requiring both bacterial and epi-

thelial cell energy production and was sensitive to inhibitors of microfilaments and microtubules, demonstrating that epithelial cell cytoskeletal rearrangements are involved in bacterial entry. *P. gingivalis* but not epithelial cell protein synthesis was necessary for invasion. Invasion within the epithelial cells was not blocked by inhibitors of protein kinase activity. Invasion was inhibited by protease inhibitors, suggesting that *P. gingivalis* proteases may be involved in the invasion process. Low-passage clinical isolates of *P. gingivalis* invaded with higher efficiency than the type strain. Serum-inhibited invasion of the type strain had no effect on the invasion of a clinical isolate.

P. gingivalis has also been observed to replicate and persist within KB epithelial cells *in vitro* (244). Intracellular counts decreased during the first 24 h and showed a threefold increase during the second day, indicating intracellular multiplication; and after 8 days the level declined to approximately 40% of the initial invasion. The ability of *P. gingivalis* to persist and multiply within epithelial cells may constitute an important pathogenic mechanism in periodontal disease.

As discussed earlier, temperature can modulate the expression of the *P. gingivalis* fimbriae. *P. gingivalis* grown at 34°C, compared with 39°C, exhibited an increased ability to adhere to *S. gordonii* and to invade primary cultures of gingival epithelial cells. These studies indicate that the altered *fimA* expression can modulate the adherence and invasion abilities of *P. gingivalis*.

Several recent reports have examined the response of the epithelial cell to *P. gingivalis* infection. Izutsu et al. (177) observed that invasion of primary gingival epithelial cells was significantly inhibited by thapsigargin and 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, acetoxymethyl ester (BAPTA/AM) but not by EDTA or amiloride. Based on these results these investigators proposed that the release of $[Ca^{+}]$ from an intracellular store with the subsequent increase in cytosolic $[Ca^{++}]$ could be involved in the invasion process, while $[Ca^{++}]$ influx was not. Moreover, cytosolic $[Ca^{++}]$ was found to increase transiently in about 30% of gingival epithelial cells acutely exposed to *P. gingivalis* but not in unexposed cells or in cells exposed to noninvasive *E. coli*. Thus *P. gingivalis* invasion of epithelial cells appears to be correlated with the activation of $[Ca^{++}]$ -dependent host cell signaling systems.

The events related to receptor function, signal transmission and cytoskeletal rearrangements concurrent with *P. gingivalis* invasion of oral epithelial

cells *in vitro* have also been examined (343). Sandros et al. (343) were able to demonstrate that *P. gingivalis* internalization involved a receptor-mediated endocytosis pathway. Treatment of the epithelial cells with monodansylcadaverine and ouabain resulted in reduction in the number of invading *P. gingivalis*. In addition, exposure of the epithelial cells to the protein kinase inhibitor staurosporine and the tyrosine-specific protein kinase inhibitor genistein also resulted in a significant decrease in the number of invading bacteria. These latter observations suggest involvement of tyrosine phosphorylation in signal transduction during invasion. These investigators also identified a 43-kDa protein that appeared to function as a substrate for tyrosine phosphorylation subsequent to the microbial-host cell interaction. Protein kinase inhibitors resulted in a significant reduction in tyrosine phosphorylation of the 43-kDa protein. Invasion of the epithelial cells was also decreased after treatment with colchicine and nocodazole, both inhibitors of microtubule polymerization. Therefore, the bacterial-receptor interaction and the phosphotyrosine-dependent intracellular signaling trigger an internalization process involving rearrangements of cytoskeletal microtubules.

Bacterial entry into epithelial or other host cells generally elicits cytokine production, producing pro-inflammatory responses through secretion of IL-1 β , IL-6 and IL-8, which will recruit phagocytes into the area. Invasion of epithelial cells by *P. gingivalis* induces a strong IL-1 β mRNA response and this is positively correlated with the adhesive and invasive potential of *P. gingivalis*. In contrast to other reported systems, invasion of epithelial cells by *P. gingivalis* has been reported to suppress IL-8 secretion. Madianos et al. (245) demonstrated in an oral epithelial cell system using a KB epithelial cell line together with polymorphonuclear leukocytes that infection with *P. gingivalis* inhibits neutrophil migration through the epithelial layer, whereas two other common pathogens, *E. coli* and *S. typhimurium*, do not. Invasion of the epithelium was demonstrated to be a prerequisite for the inhibitory activity since the fimbria-deficient mutant DPG3 that did not invade was inactive. Invasion of *P. gingivalis* was demonstrated to attenuate the production of IL-8 and the expression of intracellular adhesion molecule-1 by epithelial cells. Darveau et al. (65) have also recently reported that invasion of gingival epithelial cells by *P. gingivalis* inhibited IL-8 accumulation. These investigators suggested that the suppression of these inflammatory mediators could result from the degradation of signaling molecules

or receptors. It is interesting to speculate that the *P. gingivalis* cysteine proteinases (gingipains) could function in some capacity in the degradation of these signaling molecules or receptors. Gingipain R has recently been demonstrated to degrade tumor necrosis factor- α *in vitro* (41); however, it remains to be determined whether other cytokines are also susceptible to attack by the gingipains.

The connective tissues of the periodontium are extremely well vascularized, allowing invading microorganisms such as *P. gingivalis* to readily enter the bloodstream. However, the ability of *P. gingivalis* to actively invade endothelial cells has not been previously examined. Recently, Deshpande et al. (71) demonstrated that *P. gingivalis* can invade bovine and human endothelial cells as assessed by an antibiotic protection assay and by transmission and scanning electron microscopy. *P. gingivalis* A7436 was demonstrated to adhere and to invade fetal bovine heart endothelial cells, bovine aortic endothelial cells, and human umbilical vein endothelial cells. Invasion efficiencies of 0.1%, 0.2% and 0.3% were obtained with bovine aortic endothelial cells, human umbilical vein endothelial cells and fetal bovine heart endothelial cells, respectively. Invasion of fetal bovine heart endothelial cells and bovine aortic endothelial cells by *P. gingivalis* A7436 assessed by electron microscopy revealed microvillus-like extensions around adherent bacteria followed by engulfment within vacuoles. Invasion of bovine aortic endothelial cells by *P. gingivalis* A7436 was inhibited by cytochalasin D, nocodazole, staurosporine, protease inhibitors and sodium azide, indicating that cytoskeletal rearrangements, protein phosphorylation, energy metabolism and *P. gingivalis* proteases are essential for invasion. In contrast, addition of rifampin, nalidixic acid and chloramphenicol had little effect on invasion, indicating that bacterial RNA, DNA and *de novo* protein synthesis are not required for *P. gingivalis* invasion of endothelial cells. Likewise, *de novo* protein synthesis by endothelial cells was not required for invasion by *P. gingivalis*. *P. gingivalis* 381 was demonstrated to adhere and to invade bovine aortic endothelial cells (0.11% and 0.1% efficiency respectively); however, adherence and invasion of the corresponding *fimA* mutant DPG3, which lacks the major fimbriae, was not detected. These results indicate that *P. gingivalis* can actively invade endothelial cells and that fimbriae are required for this process. *P. gingivalis* invasion of endothelial cells may represent another strategy utilized by this pathogen to thwart the host immune response.

In summary, the preponderance of the experi-

mental evidence indicates that *P. gingivalis* is capable of entering (invading) epithelial cells. The interaction of *P. gingivalis* with these host cells might provide a mechanism by which these invading bacteria are able to divert the protective innate host response. Since it is the innate immune response that limits the spread of invading bacteria into deeper tissues of the host by maintaining the integrity of the epithelial barrier, the ability of *P. gingivalis* to destroy this integral barrier could result in host cell and tissue destruction. The host is also capable of regulating bacterial growth and multiplication in the periodontal pocket, as well as in deeper host tissues by the intervention of polymorphonuclear leukocytes and monocytes. The exit of these protective host cells from the vasculature is regulated by several host proteins, including E-selectin, intracellular adhesion molecule-1, monocyte chemoattractant protein-1 and IL-8. Low levels of these proteins have been identified in clinically healthy periodontal tissue (67), while in diseased periodontitis sites these mediators are expressed at very high levels. Therefore, *P. gingivalis* as well as the other members of the periodontopathic microbiota are capable of modulating the local innate immune response. While *P. gingivalis* is also capable of invading endothelial cells, it is not clear how the bacterium locally modulates the expression of endothelial adhesion molecules and cytokines. Future studies should define the molecular events that occur in *P. gingivalis* invasion of these host cells and further define the relationship between the local and systemic consequences of *P. gingivalis* infection.

Genetic systems. Genetic analysis of *P. gingivalis* has been slow due to the lack of naturally occurring plasmids, bacteriophages and efficient genetic transformation systems. A number of investigators have recently taken advantage of plasmids and transposons present in the intestinal *Bacteroides* species for use in *P. gingivalis*. One of the first reports on gene transfer in *P. gingivalis* used the broad host-range vector R751 to provide transfer functions and transfer of plasmid pVAL-1 and transposon Tn4351 following conjugation from *E. coli* into *P. gingivalis* (114, 116, 370). Transfer frequencies for both elements varied between 10^{-6} and 10^{-11} , depending upon the recipient strain. The behavior of pVAL-1 and Tn4351 in *P. gingivalis* was essentially as described previously in *Bacteroides* spp. These initial studies provided initial support for the conjugal transfer of plasmid and transposon DNA into *P. gingivalis*.

Two *E. coli*-*Bacteroides* plasmid-shuttle vectors pNJR5 and pNJR12 have also been introduced into *P. gingivalis* by conjugal transfer from *E. coli* (247). The transfer frequencies were comparable to those obtained when using colonic *Bacteroides* as recipients. Both plasmids were maintained in *P. gingivalis* and could be isolated and introduced back into *E. coli*. Plasmid DNA extracted from one *P. gingivalis* pNJR12 transconjugant had an additional 1.5 kb of inserted DNA. Southern-blot analysis of *P. gingivalis* chromosomal DNA using this inserted DNA as a probe revealed the presence of multiple copies of this sequence on the chromosome. This DNA sequence was identified as an insertion sequence element and referred to as IS1126.

In a separate study, the erythromycin resistance gene on Tn4351 was also introduced into *P. gingivalis* by conjugation (165). Erythromycin-resistant transconjugants were obtained at a mean frequency of 1.6×10^{-7} from matings between *E. coli* HB101 containing R751:: ω 4 and *P. gingivalis* ATCC 33277. Southern blot hybridization analysis indicated that about half of the erythromycin-resistant *P. gingivalis* transconjugants contained simple insertions of Tn4351 and half contained both Tn4351 and R751 sequences. The presence of R751 sequences in some *P. gingivalis* transconjugants most likely occurred from Tn4351-mediated cointegration of R751, since the authors were unable to detect autonomous plasmids in these *P. gingivalis* transconjugants. The *P. gingivalis*-Tn4351 DNA junction fragments from different transconjugants varied in size. These results are consistent with transposition of Tn4351 and with insertion at several different locations in the *P. gingivalis* chromosome.

Transposon mutagenesis (Tn4351) produced mutants that exhibited simultaneous deficiency in trypsin-like protease activity and hemagglutination activity. Two major membrane-associated proteins, observed by SDS-PAGE with the parent strain, were essentially absent from the mutant strains. Immunoblot analysis indicated that these two proteins corresponded to putative hemagglutinin and hemagglutinin or protease products of *P. gingivalis*. Each mutant contained only one transposon insertion; thus, the pleiotropic phenotype resulted from single site-specific mutations.

Genco et al. (116) also reported on a system for transpositional mutagenesis utilizing the *Bacteroides fragilis* transposon Tn4351. Using Tn4351, a number of mutants were isolated and in particular two mutants were characterized further. One mutant, designated MSM-1, exhibited enhanced resistance to

polymorphonuclear leukocyte phagocytosis and killing. *P. gingivalis* MSM-1 was initially selected based on its colony morphology. MSM-1 appeared as a mucoid, beige-pigmented colony. Analysis of MSM-1 by electron microscopy and staining with ruthenium red revealed the presence of a thick ruthenium red staining layer that was twice the thickness of this layer observed in the parent strain. MSM-1 was also found to be more hydrophilic as compared with strain A7436 by hydrocarbon partitioning. Analysis of phenol-water extracts prepared from *P. gingivalis* A7436 and MSM-1 by Western blot analysis and immunodiffusion using hyperimmune sera raised against A7436 and MSM-1 revealed the loss of a high-molecular-weight anionic polysaccharide component in extracts prepared from MSM-1. *P. gingivalis* MSM-1 was also found to be more resistant to polymorphonuclear leukocyte phagocytosis and intracellular killing when compared with the parent strain as assessed in a fluorochrome phagocytosis microassay, as well as more resistant to killing by crude granule extracts from polymorphonuclear leukocytes as compared with *P. gingivalis* A7436. The increased evasion of polymorphonuclear leukocyte phagocytosis and killing exhibited by MSM-1 was thus believed to result from alterations in polysaccharide-containing antigens.

The high frequency of transposition together with the stability of the insertion indicated that Tn4351 mutagenesis would be a valuable tool for examining a variety of mutations in *P. gingivalis*. However, further characterization of several *P. gingivalis* Tn4351 transconjugants indicated that Tn4351 insertion resulted in the mobilization of several endogenous *P. gingivalis* insertion sequence elements. Simpson et al. (371) have recently reported that the integration of Tn4351 into the *P. gingivalis* chromosome can result in the mobilization of the *P. gingivalis* insertion sequence element IS1126. These investigators postulated that either transposition of IS1126 occurred following introduction of Tn4351 or that mobilization may have occurred spontaneously during laboratory passage. However, Southern blot hybridization analysis of genomic DNA from 15 independent passages demonstrated that laboratory passage did not result in the mobilization of IS1126 (371). In the study of Simpson et al. (371), characterization of a *P. gingivalis* Tn4351-generated mutant revealed that an additional copy of IS1126 had inserted 185 bp upstream of the start codon of the signal peptide of the lysine-specific proteinase gene, *kgp*. The genetic rearrangement of IS1126 upstream of the *kgp* gene resulted in undetectable transcript

levels of *kgp*. Interestingly, the promoter region of *kgp* has not previously been identified. The location of the IS1126 insertion indicated that IS1126 may have inserted into a promoter region or a putative ribosomal binding site. The area of IS1126 insertion was an AT-rich region, lending further support to the role of this region as the putative *kgp* promoter.

In the same study, and in the same mutant, a second additional copy of IS1126 was found upstream of the TonB-dependent *hmuA* outer membrane receptor. Interestingly, this mutant also exhibited an increase in *rgp2* transcription. The increase in *rgp2* transcription was postulated to result from the hemin transport defect in this *P. gingivalis* mutant. An effect on *P. gingivalis* protease activity due to inactivation of a gene involved in hemin uptake has recently been documented. Aduse-Opoku et al. (5) have reported on the identification of the *tla* gene, which is required by *P. gingivalis* for growth with low levels of hemin. A *tla* mutant produced significantly lower arginine- and lysine-specific protease activities, suggesting that a regulatory link exists between *tla* and other members of this gene family. Simpson et al. (371) also proposed that the increased level *rgp2* transcription and the correlating enhanced arginine-specific cysteine proteinase activity observed in *P. gingivalis* MSM-3 could be explained by the levels of hemin present in the organism. The inability of *P. gingivalis* MSM-3 to internalize hemin would render the organism in a perpetual hemin-limited state. Studies have previously shown that hemin modulates the expression of the proteinase activities of *P. gingivalis*, both positively and negatively (114). While the precise mechanism by which hemin may regulate gingipain expression is currently unknown, the hemin-limited state of MSM-3 may serve as a signal to up regulate the transcription of gingipains (*rgp2*), thus allowing the organism to degrade a barrage of host proteins to survive within the periodontal pocket.

Thus, it appears as though the mobilization of endogenous insertion sequence elements can influence the transcription and expression of gingipains. Several studies have shown that IS1126 often flanks the *P. gingivalis* gingipain K gene (5, 21). Mobilization of insertion sequence elements within the *P. gingivalis* genome and the modulation of gingipain expression may be a common phenomenon that renders *P. gingivalis* more capable of surviving within the unfavorable environment of the periodontal pocket.

A second insertion sequence element in *P. gingivalis* designated PGIS2 was recently identified (431)

and this insertion sequence element was also found to be mobilized following the introduction of Tn4351. A *P. gingivalis* Tn4351-generated transconjugant was found to possess a complete copy of the previously unidentified insertion sequence element, which these investigators designated PGIS2. The insertion sequence element was found to have inserted into IS4351R in Tn4351 producing PGIS2, which was 1207 bp in length with 19-bp imperfect terminal inverted repeats. The insertion resulted in a duplicated 10-bp target sequence. Southern hybridization analysis of the chromosomal DNA isolated from several *P. gingivalis* strains with a PGIS2-specific probe demonstrated that the number of copies of PGIS2 per genome varied with each of the *P. gingivalis* strains. Interestingly, computer analysis of the putative polypeptide encoded by PGIS2 revealed strong homologies to the products encoded by IS1358 from *V. cholerae*, ISA1 from *Aeromonas salmonicida* and H-rpt in *E. coli* K-12, suggesting that the introduction of Tn4351 into the *P. gingivalis* genome results in the mobilization of endogenous insertion sequence elements. The complexity of *P. gingivalis* genomic rearrangements following Tn4351 transposition thus will restrict its use for further transpositional mutagenesis for *P. gingivalis*. Future studies to generate *P. gingivalis* random mutants will have to rely on shuttle mutagenesis techniques.

Construction of *P. gingivalis* mutants by homologous recombination. The ability to construct isogenic mutants of *P. gingivalis* has enabled investigators to begin to define at the molecular level the role of specific virulence factors in *P. gingivalis* pathogenesis (213). This has been accomplished by construction of mutants in *E. coli* followed by introduction of the mutated DNA back into *P. gingivalis* by electroporation. Electroporation is an efficient mechanism for the introduction of chromosomal and plasmid DNA into *P. gingivalis* (455). For example, *P. gingivalis* has been transformed by electroporation with the *Bacteroides* plasmid pE5-2 or its derivative, pYT7. Prior to transformation, pE5-2 was transferred from *E. coli* to *P. gingivalis* strains by conjugation (mobilization with R751), and the plasmid DNA was purified from the *P. gingivalis* transconjugants. Transformation occurred when the recipient strain and the donor strain from which the plasmid DNA was purified were homologous. If they were heterologous, transformation did not take place or did so at a very low frequency. These results suggested that a restriction-modification systems were operative in *P. gingivalis*. This is in agreement with a

report on the identification of a restriction modification system in *P. gingivalis* (329). A plasmid construct for *P. gingivalis* was also constructed (pYT7) by removing an 8.0-kb *Ava*I fragment from pE5-2 that was purified from *P. gingivalis*. The plasmid pYT7 has several single-cutting restriction sites usable for gene cloning; however, pYT7 was not stable in *P. gingivalis* cells, most likely due to the fact that the *rep* gene was derived from a relatively distant species, *Bacteroides eggerthii*.

A report on a host-vector system for transformation of *P. gingivalis* was also recently described in which the plasmid vectors were first isolated from *P. gingivalis* (454). Plasmid pE5-2 was employed since it could be conjugated to *P. gingivalis*. pE5-2 DNA prepared from *E. coli* was introduced by electroporation into chemically mutagenized *P. gingivalis* cells. By this method, three putative restriction-negative clones were selected. These strains exhibited a capacity for electroporation with plasmid DNAs both from *E. coli* and from various *P. gingivalis* strains at a similar efficiency. Using one of the derivatives thus obtained, YH522 plasmids that could replicate stably in *P. gingivalis* were screened. Since no plasmids were found from *P. gingivalis*, cryptic plasmids from other species of black-pigmented oral anaerobic rods were examined for their ability to transform *P. gingivalis*. A series of plasmids constructed by ligation with pBR322 for replication in *E. coli* and the *Eco*RI-B fragment from pBF4 containing erythromycin resistance were prepared from *E. coli* and were used for electroporation of *P. gingivalis*. Among these, a recombinant plasmid containing the replicon of pYHBA1 from *P. asaccharolytica*, designated pYH400, was found to be incorporated into the restriction-negative *P. gingivalis* strain and replicated stably.

Using electroporation and various antibiotic resistant markers, a number of *P. gingivalis* mutants in specific genes have been successfully isolated. The *P. gingivalis* *fimA* gene was inactivated by a homologous recombination and examined for changes in surface properties, including production of fimbriae, adherence to human gingival fibroblasts and epithelial cells, hemagglutinating activity and surface hydrophobicity. To inactivate the *fimA* gene, a *fimA* clone was disrupted by insertion of a DNA segment containing an erythromycin resistance (*Emr*) gene. This was then delivered into *P. gingivalis* ATCC 33277 from *E. coli* K-12 using a mobilizable suicide vector, pGP704. Disruption of the *fimA* locus and disappearance of *fimA* production were confirmed by Southern hybridization with a *fimA*-specific DNA probe

and Western immunoblotting with a monoclonal antibody against the *fimA* protein, respectively. The *fimA* mutant constructed failed to express long (0.5 to 1.0 μm) fimbriae from the bacterial surface and had a diminished adhesive capacity to tissue-cultured human gingival fibroblasts and epithelial cells. Observation of the bacteria adhering to human gingival fibroblasts by scanning electron microscopy revealed that the wild-type strain had dramatic local changes in the appearance of the microvilli at the point of contact with large bacterial clumps, whereas the *fimA* mutant did not. In contrast, neither the hemagglutinating activity nor the surface hydrophobicity was changed in the *fimA* mutant. The mutant was also demonstrated in latter studies to be altered in its ability to adhere to and to invade epithelial cells (see section on invasion).

A *Tpr* deficient isogenic mutant of *P. gingivalis* was generated by homologous recombination between *P. gingivalis* chromosomal DNA and a suicide plasmid carrying the cloned gene disrupted by insertion of an erythromycin resistance gene. Gelatin substrate zymography showed that cell extracts of the mutant lacked a protease band that migrated with an apparent molecular mass of 80 kDa. Western immunoblots of the cell extracts indicated the loss of an antigen with a similar mass.

An isogenic mutant, G-102 of *P. gingivalis* 381 deficient in Arg-gingipain cysteine protease activity, was constructed by Tokuda et al. (416). The mutant displayed both reduced protease activity and significantly reduced hemagglutination activity compared with the wild-type strain. These studies provided genetic evidence for the recently proposed structural relationship between Arg-gingipain and the hemagglutinin activity of *P. gingivalis* strains.

The construction of a *P. gingivalis* *tla* mutant enabled a function in hemin transport to be ascribed to this gene. Similarly, a *P. gingivalis* *hmuA* mutant was recently constructed (372) allowing for a function to be ascribed to this hemin-regulated gene. A recent report using transposon-induced mutagenesis has clearly documented a relationship between pigmentation and trypsin-like activity as well as hemagglutination activity (168).

Reporter genes. Several reporter genes for transcriptional fusions have been developed and should be useful for studies of gene regulation in *P. gingivalis*. Several investigators have constructed fusions with *P. gingivalis* genes using the *lacZ* gene. These include fusions to the *fimA* gene and the *hemR* gene. Whitehead (439) previously cloned a gene encoding

a bifunctional xylosidase/arabinosidase (XA) enzyme from *Bacteroides ovatus* V975 as part of a xylan-inducible operon. *B. ovatus* is a normal inhabitant of the human intestinal tract and is one of the few *Bacteroides* species capable of degrading xylan, a major component of fiber in the diet. The XA gene cloned in *E. coli* is under transcriptional regulation in *E. coli* by the *lac* promoter, and both activities can be induced with isopropylthio- β -galactoside. The XA gene was subcloned into *E. coli* and *Bacteroides* shuttle vectors and introduced by conjugation into *P. gingivalis*. The characteristics of the XA reporter system are low background or total lack of arabinosidase and xylosidase activities in *P. gingivalis* and the ease of enzymatic assays. In addition, bacterial colonies can be screened directly on agar plates by fluorescence with methylumbelliferyl derivatives as substrates for either enzymatic activity.

Genomic rearrangements. In addition to the recent reports of mobilization of endogenous insertion sequence elements (371, 431), two recent studies (279, 300) provide data supporting genomic rearrangements as a mechanism for genetic variability in *P. gingivalis*. A comparison of the *rgpB* gene and the *rgpI* gene, one of the *rgpA*-equivalent genes, revealed that their gene structures were very similar to each other, except that the *rgpB* gene did not possess most of the hemagglutinin domain present in the C-terminal region of the *rgpI* gene and provided strong evidence for homologous recombination between the proteinase domain regions of the two *rgp* genes. The presence of nonreciprocal recombination in *P. gingivalis* was experimentally proven using suicide and integration plasmid systems. The results provide one of the hypothetical scenarios of the generation of the two *rgp* genes: that is, they have been generated through the duplication of an ancestor *rgp* gene, insertion of the hemagglutinin domain region into one copy of the two resulting *rgp* genes (or deletion of the region from one *rgp*) and homologous recombination between the proteinase domain regions of the two *rgp* genes. It is interesting to speculate that this type of rearrangement may be responsible for the genetic diversity of this family of genes.

Comparison of the nucleotide sequences of *rgpI* and *kgp* indicates that a majority of the C-terminal sequences of these genes are identical. It has been suggested that recombinational rearrangement such as transposition or gene conversion may have occurred in this nucleotide region between *kgp* and *rgpI*. At least two other DNA regions on the *P. gingivalis* chromosome that may encode for other hem-

agglutinins share homology with this region, suggesting that these DNA regions may have also taken part in this recombinational event. It is also possible that these DNA regions may have been supplied from the chromosomal DNA of other *P. gingivalis* cells (horizontal gene transfer). Gene conversion type recombination has been observed in *P. gingivalis* (300), and thus it is reasonable to postulate that recombination between *P. gingivalis* *rgp1* and *kgp* could occur by such a mechanism. There are numerous examples in which gene conversion results in antigenic variation of genes in a variety of bacterial pathogens (356). The ability to undergo antigenic variation provides pathogens with protection from immune challenge by their hosts.

A recent report (139) on the identification of the *P. gingivalis* *hagA* gene lends further support to genomic rearrangements as a mechanism to yield diversity in *P. gingivalis* genes. Multiple hemagglutinin genes have been identified from *P. gingivalis* 381. Han et al. (139) cloned and sequenced the *hagA* gene, which was found to be 7887 bp in length, encoding a protein of 2628 amino acids with a molecular mass of 283.3 kDa. Within its open reading frame, four large, contiguous, direct repeats (varying from 1318 to 1368 bp) were identified. The repeat unit (HArep), which was assumed to contain the hemagglutinin domain, was also present in other recently reported protease and hemagglutinin genes in *P. gingivalis*. The presence of multiple repeat units may provide a means for *hagA* to undergo rearrangements (duplications or deletions of a repeat unit) and thus antigenic variation.

In addition to gene conversion, evidence also exists for transposition as a possible mechanism for recombinational rearrangements in *P. gingivalis* (21, 371). It is well established that genes proximal to an insertion element can be subjected to transposition or deletion (7, 109). Interestingly, the *P. gingivalis* endogenous IS element IS1126, was found 3' to the *prtP* gene (*kgp* homolog) in strain W12, and it was suggested that this insertion sequence element could serve as one end of a composite transposon (21). Recombination within the locus encoding gingipain K could thus have occurred via a transposition event. In support of this contention, Simpson et al. (371) found that IS1126 is capable of mobilization in *P. gingivalis*. Analysis of a *P. gingivalis* strain in which IS1126 was mobilized indicated that IS1126 had inserted directly upstream of the *kgp* gene. The demonstration of the mobilization of the second endogenous *P. gingivalis* insertion element PGIS2 in the *P. gingivalis* chromosome has also been discussed.

Thus, the ability of insertion sequence elements to be mobilized within *P. gingivalis*, together with the finding that these elements are commonly found flanking the *kgp* locus, indicates that the variability in this locus may be due to genomic rearrangements facilitated by transpositional events.

Further support of an active recombination system in *P. gingivalis* comes from the isolation of the *P. gingivalis* *recA* homolog (96). These investigators employed degenerate oligonucleotide primers and were successful in amplifying a region of the *recA* homolog from *P. gingivalis* W83. The PCR fragment was able to identify a recombinant lambda DASH phage (L10) carrying the *P. gingivalis* *recA* homolog and was identified as a 1.02-kb open reading frame (341 amino acids), with the predicted amino acid sequence strikingly similar (90% identical residues) to the *RecA* protein from *B. fragilis*. No SOS box characteristic of *LexA*-regulated promoters was identified. Insertional inactivation of the cloned *P. gingivalis* *recA* gene employing *ermF*-*ermAM* antibiotic resistance resulted in the formation of a *recA*-deficient mutant (FLL33), which was significantly more sensitive to ultraviolet irradiation than the wild-type strain W83. Both W83 and FLL33 showed the same level of virulence in *in vivo* experiments using a mouse model, suggesting that the *recA* gene in *P. gingivalis* W83 might play a role in DNA repair. However, inactivation of this gene did not alter the virulence of *P. gingivalis* in the mouse model.

The ability to genetically manipulate *P. gingivalis* has already provided some important information on the role of this putative periodontopathogen in host virulence (that is, periodontitis). With the analysis of the complete *P. gingivalis* genome sequence, the *in vivo* role of this bacterium with a host will be possible to study. The evidence to date clearly indicates that *P. gingivalis* is able to modulate gene expression through both genomic rearrangement and transposition – two genetic manipulations that could contribute to the ability of this bacterium to evade host detection and compromise the host in both the local environment of the periodontium as well as systematically, such as in the cardiovascular system.

Animal models to study *P. gingivalis* pathogenesis and periodontal disease

A number of different animal models have been used to evaluate the complex interactions between *P. gin-*

gingivalis and the host and have been an important research tool to study the pathogenesis of *P. gingivalis*-mediated periodontal diseases. The host response to periodontopathic organisms plays a major role in the outcome of periodontal diseases. Bacteria trigger inflammatory host responses, which, along with the direct destructive effects of bacteria, cause the majority of the tissue destruction. Numerous investigations have elucidated important aspects of the complex interactions of *P. gingivalis* with the intact host through the use of various animal models.

An important feature of any animal model used to study human infectious diseases is that it should simulate an infectious process in humans while mimicking the natural pathogenesis to the greatest extent possible. Clinical landmarks of periodontal infections include growth of epithelial attachment, loss of connective tissue attachment and bone loss. Animal models have been developed to examine various aspects of these interactions using primates (monkeys and baboons), dogs, rodents (rats, mice and rabbits), and sheep. Primate, canine, mouse, and rat models are suitable for assessing invasion by *P. gingivalis* in periodontal tissue and in assessing the ability of specific *P. gingivalis* mutants to colonize and cause bone loss (241). Primate models have been utilized primarily to examine the host response induced to *P. gingivalis* infection and to assess the immune potential of various *P. gingivalis* virulence factors.

Pivotal studies reported by Holt et al. (163) demonstrated the causative link between *P. gingivalis* and periodontal disease in a monkey model of periodontal disease. To directly assess the role of *P. gingivalis* in the progression of periodontal disease, a rifampin-resistant *P. gingivalis* strain was implanted in *M. fascicularis*, and a direct connection between the implantation of this strain and the development of periodontitis was observed. Rapid and significant bone loss, as well as increases in antibody levels to *P. gingivalis*, occurred after implantation of this strain.

The roles of tumor necrosis factor- α and IL-1 β in the process of periodontal destruction have also been assessed in the *M. fascicularis* primate model (17). Periodontal destruction was induced using ligatures soaked with *P. gingivalis*, and soluble receptors to tumor necrosis factor and IL-1 were injected in the experimental sites while control sites received only saline. The authors found that inhibition of tumor necrosis factor and IL-1 resulted in a reduction in inflammatory cell recruitment and bone loss by 60%, suggesting that tumor necrosis factor and IL-1 activity is an important factor in the pathologic pro-

cess of periodontitis. Several different species of monkeys, *M. fascicularis* (cynomolgus monkey), *Macaca mulatta* (rhesus monkey) and *Macaca nemestrina* (pig-tailed monkey), have been used for studies of periodontal disease. Primate models have been used to examine the disease process and host response to *P. gingivalis* and, ultimately, for vaccine testing. It has been shown that the *M. nemestrina* species exhibits clinical features of periodontitis similar to that found in humans. *M. nemestrina* also harbor subgingival microflora that may cause disease in humans and express serum IgG antibodies to *P. gingivalis* antigens as a consequence of colonization by *P. gingivalis*. Thus, the *M. nemestrina* species may be a useful model for studying interactions between the host and periodontal microbiota (319).

Primate models have also been used to study how immunization with *P. gingivalis* and *P. gingivalis*-specific components affects the progression of ligature-induced periodontitis. Immunization of *Saimiri sciureus* (squirrel monkey) monkeys with *P. gingivalis* was demonstrated to result in increased levels of anti-*P. gingivalis* serum IgG and a significant reduction in the colonization of *P. gingivalis* in the gingival crevice (255). Immunization studies in the *M. fascicularis* primate model with intact *P. gingivalis* was also shown to result in significant, specific, and sustained antibody responses to *P. gingivalis* (119, 162). Holt et al. (162) immunized *M. fascicularis* with cell envelope antigens prepared from *P. gingivalis*. Following immunization with *P. gingivalis*, antibodies produced to the surface antigens were demonstrated to interfere with the emergence of *P. gingivalis*. In addition, this interference resulted in increased growth and emergence of other black-pigmented bacteria. However, further studies are required to define the role of a specific antibody response to defined *P. gingivalis* antigens in these primate models.

The functional activity of nonhuman primate (*M. fascicularis*) antibodies to *P. gingivalis* was examined by Anderson et al. (14), who analyzed normal or baseline sera, sera obtained from animals with ligature-induced periodontitis and sera obtained from animals immunized with formalinized *P. gingivalis*. Increased antibody levels were found in the sera after immunization with formalin-killed *P. gingivalis*. In addition, the uptake of *P. gingivalis* by polymorphonuclear leukocytes was significantly enhanced in immunized animals. A functional antibody that enhanced direct killing and the ability of polymorphonuclear leukocytes to phagocytize *P. gingivalis*, as well as positively influenced polymorphonuclear leukocyte activation, was observed in immunized animals.

The *M. fascicularis* primate model was utilized by Persson et al. (320), who demonstrated an inverse correlation of antibody titer and colonization of *P. gingivalis*. It was demonstrated that the percentage of *P. gingivalis* in the subgingival microbiota decreased as the titer increased. This finding supports the concept that the humoral immune response may have favorable effects on the colonization and growth of *P. gingivalis*.

An *M. mulatta* model has been utilized to examine the changes that occur in cyclooxygenase metabolites during periodontitis (287). Three of four groups were treated with flurbiprofen, a potent cyclooxygenase inhibitor that has been shown to significantly retard attachment loss. Compared to baseline values, a 3-fold increase was seen in the crevicular fluid levels of prostaglandin E_2 and thromboxane B_2 in untreated animals at 3 months after initiation of ligature-induced periodontitis. At 6 months, a dose-dependent inhibition of prostaglandin E_2 and thromboxane B_2 was seen after treatment with flurbiprofen, implicating cyclooxygenase products in the pathogenesis of destructive disease.

A number of studies have described *P. gingivalis* mediating periodontal disease in rats. The periodontal anatomy, development and composition of plaque, and the histopathology of periodontal lesions in rats is similar to that found in humans (205). This feature has been utilized to examine the role of specific *P. gingivalis* bacterial components in tissue destruction and bone loss. A germ-free rat model in which *P. gingivalis* is infected orally by gavage has been utilized to examine the involvement of the *P. gingivalis* fimbriae in colonization and bone loss (205). A *P. gingivalis* *fimA* mutant was significantly less able to cause periodontal bone loss in this model (246). Immunization with the fimbrial protein was also demonstrated to protect against periodontal tissue destruction following challenge with wild-type *P. gingivalis* in the germ-free rat model.

Wang & Stashenko (432) used a rat model to identify the mediators that stimulate bone resorption following infection. This model was designed so that active (rapid) and chronic (slow) phases of bone destruction could be distinguished. Pulpal exposure followed by infection from the oral environment was used to induce bone resorption. These studies revealed that IL-1 α was the primary mediator of bacterial infection in this model.

A rat model has been used to study the effects of diabetes on periodontal disease and to characterize the different gingival cytokine profiles in response to *P. gingivalis*-induced periodontal disease (74). Dia-

betes was induced chemically by streptozotocin administration. A significant increase in the levels of platelet-derived growth factor β and IL-1 β was observed in the nondiabetic periodontal disease model. However, these increases did not occur in the diabetes model, and they were prevented in the model with periodontitis superimposed on diabetes. These results demonstrated that the normal host response to periodontitis is altered by the presence of diabetes through the prevention of periodontitis-induced increases in IL-1 β and platelet-derived growth factor β .

The effects of *P. gingivalis* on adverse pregnancy outcomes have been studied in hamster models. Collins et al. (56) found that lipopolysaccharide from *P. gingivalis* significantly reduced the fetal weight, suggesting that maternal exposure to *P. gingivalis* lipopolysaccharide can have harmful effects on the developing fetus. It has also been shown that fetal growth retardation and embryo lethality were significantly correlated with increases in prostaglandin E_2 and tumor necrosis factor- α occurring after *P. gingivalis* challenge (57).

Perhaps the most widely used animal model to examine *P. gingivalis* pathogenesis is the mouse. Baker et al. (18) developed a mouse model to examine oral infection with *P. gingivalis*. In this model, immunocompetent and severe combined immunodeficient mice were infected orally (by gavage) with *P. gingivalis*, and bone loss was monitored. *P. gingivalis* was cultured from the oral cavity throughout the experiment, and *P. gingivalis*-specific antibodies were detected in mouse sera. These investigators postulated that, due to the mutation in severe combined immunodeficient mice (lacking T and B lymphocytes), bone loss should be reduced or prevented if T and/or B cells participate in periodontal destruction. However, alveolar bone loss was observed following infection with *P. gingivalis*, indicating that bone resorption could occur in the absence of T and/or B cells. The mouse model of oral *P. gingivalis* infection has also recently been used in IL-2, IL-4 and interferon- γ knockout mouse strain to define the role of these mediators in *P. gingivalis* pathogenesis. This mouse model has also been used to define the virulence potential of various strains of *P. gingivalis* (personal communication, Pam Baker).

A murine abscess model has been used by numerous investigators to examine the role of various *P. gingivalis* virulence factors in pathogenesis. Following subcutaneous infection with *P. gingivalis*, mice develop primary lesions at the site of infection as well as secondary lesions distal from the site of in-

fection. The mouse abscess model has been used to examine the immunogenic potential of *P. gingivalis* whole cells as well as defined bacterial components. For example, immunization with *P. gingivalis* polysaccharide was demonstrated to reduce the severity of primary and secondary abscess formation; however, immunization did not completely prevent invasive infection with *P. gingivalis*, indicating that an immune response to polysaccharide in itself is not sufficient for protection to *P. gingivalis* infection (349). The murine abscess model has also been used to determine the phenotype of lymphocytes and macrophages recruited into periodontal lesions following immunization with *P. gingivalis* ATCC 33277. Acid phosphatase-positive and phagocytic macrophages were predominantly found in the lesions of immunized mice as assessed at day 4 after challenge. These cells were found primarily on the periphery of healing lesions by day 7. The authors suggested that the protective immune response to *P. gingivalis* seen in this model is consistent with a strong immune response, which may be controlled by T cells (110). The murine subcutaneous chamber model has also been used to examine the virulence potential of *P. gingivalis* mutants, the host response to infection and the cellular interaction between *P. gingivalis* and polymorphonuclear leukocytes. This model allows for continual sampling from subcutaneous chambers to examine the modulation of specific virulence factors *in vivo* and to assess the polymorphonuclear leukocyte infiltrate in response to *P. gingivalis* infection. Genco et al. (115) have confirmed that *P. gingivalis* strains that are resistant to polymorphonuclear leukocyte phagocytosis and killing *in vitro* are also resistant to polymorphonuclear leukocyte phagocytosis and killing *in vivo*. *P. gingivalis* mutants upregulated in the expression of gingipain R2 are highly virulent in this model (Genco CA, unpublished data). This model has also been used to demonstrate a protective immune response following immunization with gingipains R1 and R2 (113).

Several studies have defined the role of the *P. gingivalis* proteinases in the abscess model. Kesavalu et al. studied the role of the *P. gingivalis* proteases using the mouse abscess model to assess their role in soft tissue destruction (196, 197). The Kesavalu et al. (196) study employed several inbred and outbred mouse strains with altered immune characteristics. *P. gingivalis* W50 challenge of these animal strains resulted in the formation of a subcutaneous skin lesion of approximately 400 mm² diameter. However, natural killer cell-deficient mice (C57BL/6J-BgJ)

formed a lesion that was significantly larger and more severe than the other mouse strains studied. Secondary challenge of these animals after healing of the primary lesions revealed a delayed onset as well as a significantly decreased lesion size compared to control animals. However, all of the immunodeficient animals were not capable of developing any immune protection to the secondary challenge, suggesting that the protection afforded to the normal immune animals to *P. gingivalis* lesion formation is mediated by innate immune mechanisms, such as polymorphonuclear leukocytes and natural killer cells. These investigators also found that the production of trypsin-like protease enzyme activity was related to the size and type (localized or spreading) of the lesion as well as to the death of mice (197). More recently, *P. gingivalis* double mutants defective in the arginine-specific cysteine proteinases (gingipain R1 and gingipain R2) were found to exhibit a decrease in virulence as assessed by abscess formation in this model (279).

A murine model to study the early cellular and molecular events associated with the host effects of *P. gingivalis* lipopolysaccharide infection was utilized by Reife et al. (336). Mice were given intramuscular injections of *P. gingivalis* lipopolysaccharide or *E. coli* lipopolysaccharide, and the muscles were evaluated after 4 h. Muscles injected with *E. coli* lipopolysaccharide showed higher expressions of mRNA for E- and P-selectins as compared with *P. gingivalis* lipopolysaccharide-injected muscles. There was also an absence of monocyte chemotactic protein 1 and fibroblast-induced cytokine mRNAs in *P. gingivalis* lipopolysaccharide-injected muscles. These results suggested that *P. gingivalis* has a low biologically reactive lipopolysaccharide, which may allow it to colonize and cause chronic disease, and are consistent with *in vitro* studies in which *P. gingivalis* lipopolysaccharide was observed to serve as an antagonist for E-selectin expression from endothelial cells (66).

Amar et al. (13) used a murine model to determine the role of two distinct receptors of tumor necrosis factor, tumor necrosis factor receptor-1 and tumor necrosis factor receptor-2, in lipopolysaccharide and tumor necrosis factor-induced inflammatory skin necrosis tumor necrosis factor receptor-1, tumor necrosis factor receptor-2, tumor necrosis factor receptor-1/tumor necrosis factor receptor-2 and tumor necrosis factor lymphotoxin- α deficient mice were used for this study. The authors found that tumor necrosis factor receptor-1 mediated the destructive effects of tumor ne-

crosis factor and that endogenous tumor necrosis factor may amplify the destructive effects of exogenous tumor necrosis factor.

Rabbits have also been used to examine the growth of *P. gingivalis* in chamber implants. Dahlén & Slots (64) studied the interactions between *P. gingivalis* and systemic antibodies in a rabbit subcutaneous chamber infection model. Using the tissue cage technique, infectivity was evaluated in rabbits immunized against *P. gingivalis* and nonimmunized rabbits. Compared with nonimmunized rabbits, weaker tissue reactions were observed in immunized rabbits as well as a significant reduction in bacterial counts following challenge with live *P. gingivalis*. These studies demonstrated that the pathogenic potential of periodontal pathogens could be modulated by the immune system as a function of inflammatory cell mechanisms and/or the systemic antibody response.

The molars, premolars, periodontium and metabolic rate of sheep are similar to that found in humans, and skeletal problems, such as bone loss, occur in aging sheep (173). The features of *P. gingivalis*-induced periodontal disease observed in sheep, such as epithelial infiltration, collagen breakdown and bone resorption, were similar to that found in humans with rapidly destructive forms of periodontal disease. In addition, significantly higher levels of IgG antibody reactive against *P. gingivalis* antigens were seen in sheep with periodontitis compared with those without disease, which is also similar to that found with some types of human periodontal disease. Although these studies indicate that the sheep model may be suitable for *in vivo* studies, the pathogenesis of *P. gingivalis* has not been examined in this model.

There is no doubt that the use of animal models to define the interaction between *P. gingivalis* and host cells is essential. Importantly, however, while each animal model will provide important information relevant to the role of this bacterium in compromising a specific host, it will, for the most part, only mimic that which occurs in the human condition. The factors affecting the choice of an animal model, such as animal size, cost, ease of manipulation, and how closely the model describes the pathogenic events that occur in humans, will continue to modify the choice of animals. However, the information already obtained from the well characterized small animal models (mice) and larger animals (primates) has provided important information relevant to the pathogenesis of *P. gingivalis*-mediated periodontal diseases and their sequelae.

Conclusions

The years of investigations into the clinical and biological events in the conversion of a healthy periodontal environment to one characterized by inflammation, bone and tissue destruction has been extensive and scientifically informative. Essentially all of these studies invoked the participation of plaque in this disease progression. Healthy oral tissues were free of plaque, devoid of inflammation and clinically "healthy". Disease progression was characterized by the formation of plaque, with the benchmark experiments demonstrating the association between oral hygiene, plaque formation and the growth and emergence of large numbers of different bacterial species. Bacterial "load" versus "bacterial type" next became the prevailing paradigm for health versus disease transitions, and for many years this paradigm prevailed – more plaque, more disease; less plaque, less disease. However, with the remarkable studies of several laboratories, especially those of Socransky at the Forsyth Dental Center, and later by Moore and Holdeman, it became clear that, while bacterial load was important, it was the composition of the plaque that was more than likely responsible for the maintenance of oral health and the progression and establishment of disease. The literature is replete with reports of the types and the distribution of gram-positive and gram-negative bacteria in the progression of the various bacterially mediated oral diseases. In oral health, there is a preponderance of gram-positive microorganisms occupying the healthy sulcus. The majority of these bacterial species are aerobic, microaerophilic, saccharolytic species, which during their growth and metabolism produce large amounts of organic end-products that produce a complex plaque matrix. This matrix is only partially pervious to oxygen, and as such, continued growth of these supragingival plaque bacteria reduces the oxygen tension to levels that permit resident gram-negative microaerophilic species to grow and emerge in the bacterial population. This conversion of an "aerobic plaque" to an "anaerobic plaque" is accompanied by a shift in oral health to tissue inflammation (gingivitis) and eventually periodontal disease (periodontitis) with an associated loss of tissue and bone integrity. In the studies of this health to gingivitis to periodontitis transition, a remarkable observation was discovered: of the projected hundreds of different species that eventually occupied the periodontal environment, only a very few were members of what has become known as the "periodontopathic microbiota". These

include (to date!): *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *Prevotella nigrescens*, *B. forsythus*, *C. rectus*, *F. nucleatum*, *Peptostreptococcus micros*, *E. corrodens*, *T. denticola* and *Treponema* spp. *P. gingivalis* possesses all of the biological and chemical characteristics that make it an important member of this periodontopathic microbiota – it is essentially absent in oral health, is a predominant member of the subgingival microbiota in disease and possesses and “excretes” numerous potentially toxic virulence factors. *P. gingivalis* also produces many cell components and macromolecules that have been proposed to function as virulence factors. These factors include the lipopolysaccharide, outer membrane and its component proteins (outer membrane vesicles), fimbriae and the numerous end-products of metabolism, especially the proteinases. While the other periodontal pathogens also possess potentially harmful virulence factors, and their presence within the confines of the periodontal environment varies as a function of that environment, studies of *P. gingivalis* reveal that, with very few exceptions, this bacterium is a highly pathogenic and virulent member of the subgingival plaque microbiota. *P. gingivalis* produces numerous fimbriae that appear to function similarly to that of the other host-associated gram-negative bacteria. The *P. gingivalis* fimbriae are constructed of fimbrillin subunits that form into the mature 67-kDa fimbriae. They are involved in the interaction of *P. gingivalis* with other bacterial species and with both soft and hard host tissues.

Identical to gram-negative prokaryotes, *P. gingivalis* synthesizes a lipopolysaccharide. While the lipopolysaccharide from this oral pathogen is chemically different from the other classical lipopolysaccharides, and these differences are reflected in its relatively low endotoxicity, this low endotoxicity may reflect the ability of this bacterium to grow and colonize in a host. In tissue culture studies, the *P. gingivalis* lipopolysaccharide does function as a significant cytotoxin as well as inducer of several host-derived cyto- and chemokines.

Major emphasis during the past several years has concentrated on the isolation and characterization of selected excreted or secreted macromolecules from *P. gingivalis* with periodontopathic potential. While *P. gingivalis* produces a large number of hydrolytic, lipolytic and proteolytic enzymes, the proteins known as “proteinases” have received considerable attention. While the function of these proteinases *in vivo* still remains to be unequivocally determined (see below), these *P. gingivalis* protein-

ases have been found to exhibit significant *in vitro* activity against such host proteins as host proteinase inhibitors, immunoglobulins, iron-transport and iron-sequestering proteins, matrix proteins, bactericidal proteins and peptides, and the proteins involved in coagulation, complement activity and kallikrein and kinin activity. The best studied of the *P. gingivalis* proteinases are the cysteine proteinases, or the “gingipains”, with specificities for cleavage after arginine and lysine residues. Hence, the arginine (Arg-) and lysine (Lys-) cysteine proteinases. Gingipain R1 and gingipain R2 (gingipain K) describe these cysteine proteinases. There is increasing excellent evidence that the gingipains are important proteins in the clinical events of periodontitis. In several mouse studies, inactivation of these proteinases prior to *P. gingivalis* infection results in the attenuation of its virulence capability. Similarly, immunization of mice with gingipain R protects the animals from subsequent *P. gingivalis* challenge. Further, in both mice and nonhuman primate experiments, immunization with gingipain peptides results in the generation of an immune responses that, in mice, are protective from *P. gingivalis* challenge, while in the nonhuman primate the immunization did not interfere with the emergence of *P. gingivalis* in ligated sites but did result in significant differences in bone loss from ligated sites. Thus, the gingipains may play a central role in periodontal bone and tissue destruction, and interference with the formation or activity of this proteinase may be capable of altering the periodontopathic response.

Two important experimental areas have recently emerged in studies of the role of *P. gingivalis* and several of the other periodontal bacteria: the environmental regulation of virulence expression and the use of animal models. The preponderance of the experimental evidence indicates that *P. gingivalis* is capable of entering (that is, invading) epithelial cells. The interaction of *P. gingivalis* with these host cells might provide a mechanism by which these invading bacteria are able to divert the protective innate host response. Since it is the innate immune response that limits the spread of invading bacteria into deeper tissues of the host by maintaining the integrity of the epithelial barrier, the ability of *P. gingivalis* to destroy this integral barrier could result in host cell and tissue destruction. The host is also capable of regulating bacterial growth and multiplication in the periodontal pocket, as well as in deeper host tissues by the intervention of polymorphonuclear leukocytes and monocytes. The exit of these protective host cells from the vasculature is regulated by

several host proteins, including E-selectin, intra-cellular adhesion molecule-1, MCP-1 and IL-8. Low levels of these proteins have been identified in clinically healthy periodontal tissue (65), while in diseased periodontitis sites these mediators are expressed at very high levels. Therefore, *P. gingivalis* as well as the other members of the periodontopathic microbiota are capable of modulating the local innate immune response. While *P. gingivalis* is also capable of invading endothelial cells, it is not clear how the bacterium locally modulates the expression of endothelial adhesion molecules and cytokines. Future studies should define the molecular events that occur in *P. gingivalis* invasion of these host cells and further define the relationship between the local and systemic consequences of *P. gingivalis* infection.

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